INTESTINAL HUMORAL IMMUNITY IN MAN:

IgA AND ANTI-SALMONELLA ANTIBODIES

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DECLARATION

I declare that this thesis has been composed by myself and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

Jamal A Sallam

DEDICATION

To the memory of my father

ACKNOWLEDGMENT

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SUMMARY

Studies of gut immunity must be carried out on intestinal fluid and jejunal biopsies. Recent work from Edinburgh has shown that the Whole Gut Lavage (WGL) technique is a non-invasive, direct and reliable method of obtaining intestinal fluids. My thesis describes the use of WGL technique in a variety of studies of gastrointestinal mucosal immunity. I started by confirming, in a serial lavage study, the validity of taking any clear fluid stools passed per rectum to be representative of the rest of the lavage effluent. The effect of the newly licensed oral live typhoid vaccine Ty21a on gut immunity was investigated in a group of 22 healthy British volunteers. Later on, the intestinal immune responses to naturally-acquired salmonella infection were investigated in a group of patients who had had the infection within the preceding 12 months. Results obtained in these studies were compared with results obtained from healthy individuals, patients with inflammatory bowel disease (IBD) and African children from Sierra Leone. Because previous studies had shown that cigarette smoking modifies the systemic and local immune responses and produces a decrease in the concentration of salivary IgA of smokers compared to control non-smokers, I investigated further the effect of heavy smoking and non-smoking in healthy volunteers on gut immunity and the effect of administration of the live oral vaccine Ty21a on the intestinal mucosal immune responses of smokers and non-smokers. In the course of the above studies, I found that there were patients who had very low or absent intestinal IgA but

had normal levels of IgA in the serum. Therefore, I investigated further this phenomenon by counting plasma cells in the lamina propria of intestinal biopsies from patients with "intestinal IgA deficiency " and normal controls using image analysis.

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ABBREVIATIONS

α-1-AT	alpha-1- anti-trypsin
AIDS	acquired immunodeficiency syndrome
APCs	antigen-presenting cells
BSA	bovine serum albumin
B-WC	B-subunit and whole cell (of cholera vaccine)
CD	Crohn's disease
CMI	cell-mediated immunity
ConA	Concanavalin A
CSF	colony-stimulating factor
DEA	diethanolamine
DM	diabetes mellitus
DNA	deoxyribonucleic acid
EBV	Ep stein-Barr virus
E. Coli	*Escherichia coli*
EDTA	ethylene diamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
GALT	gut-associated lymphoid tissue
GIU	gastrointestinal unit
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigens
HPV	human papilloma virus
HT	high tar
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ICAM1	intercellular adhesion molecule 1
IELs	intraepithelial lymphocytes

IFN-γ	interferon gamma
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1, 2 etc.	interleukin 1, 2 etc.
IL-2R	interleukin 2 receptor
LPS	lipopolysaccharide
LT	low tar
MALT	mucosal-associated lymphoid tissue
M cells	microfold cells
MDCK	Madin Darby canine kidney
MHC	major histocompatibility complex
mIgA	membrane-bound IgA
MNCs	mononuclear cells
mRNA	messenger ribonucleic acid
NBCS	new born calf serum
NK	natural killer
OD	optical density
OPV	oral poliovirus vaccine
PAF	platelet activating factor
PAH	polycyclic aromatic hydrocarbon
PAMs	pulmonary alveolar macrophages
PBLs	peripheral blood lymphocytes
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfc	plaque-forming cell
PGE_2	prostaglandin E_2
PHA	phytohaemagglutinin
PIgA	polymeric IgA

PIgR	polymeric immunoglobulin receptor
PMSF	phenylmethylsulfonyl fluoride
PNP	para-nitrophenyl phosphate
PPs	Peyer's patches
Rc mutant	rough mutant type c
RIA	radioimmunoassay
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolution per minute
S	sample
SBTI	soybean trypsin inhibitor
SC	secretory component
SD	standard deviation
SIgA	secretory IgA
SLE	systemic lupus erythematosus
Spp.	species
SRBCs	sheep red blood cells
S. typhi	*Salmonella typhi*
S. typhimurium	*Salmonella typhimurium*
TAB	typhoid, paratyphoid A and paratyphoid B vaccine
TCA	trichloroacetic acid
TGF-β	tumour growth factor β
Th	T helper
TIgA	total IgA
TIgG	total IgG
TIgM	total IgM
TNF	tumour necrosis factor
tRNA	transfer RNA
UC	ulcerative colitis
UDP	uridine diphosphate
V. Cholerae	*Vibrio Cholerae*

WGH	Western General Hospital
WGL	whole gut lavage
WHO	World Health Organization
WSF	water soluble fraction

PRESENTATIONS AND PUBLICATIONS BASED ON THIS THESIS

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Chapter I

INTRODUCTION

Foreword

The intestinal immune system is largely independent from the systemic immune system and the process of obtaining fluids directly from the gut is regarded as the optimal method or the " gold standard " for the investigation of the local intestinal immunity. However, investigations of the local intestinal immunity have been hampered by the difficulty of obtaining direct access to the intestinal secretions.

Extensive studies into the local intestinal immune system have been carried out both in man and animals, extrapolating determinations of levels of antigen-specific antibodies in serum, colostrum, saliva or antibody production *in vitro* by circulating peripheral blood lymphocytes (PBL) to the gut. However, indirect measurements of the local immune response do not necessarily reflect the findings at the intestinal level and studies showed that systemic immune responses are not correlated with the intestinal immune responses.

Repeated intestinal intubation to obtain intestinal secretions for the measurement of immunoglobulins and specific antibodies concentrations have been carried out as a direct approach to the local intestinal immune system. However, repeated intestinal intubation is inconvenient for the patient, invasive, labour-intensive and a time-consuming procedure. Furthermore, repeated intestinal intubation is not suitable for the very young or very old individuals. In addition, secretions obtained from the jejunum may not be representative of the whole gut.

Whole gut lavage (WGL) has recently been available for use in man and was found to be safe, reliable, non-invasive and a direct approach to study the intestinal mucosal immunity. The activity of protease enzymes, found in large amount in the human intestinal secretions, could be prevented by treating the specimens with protease inhibitors.

Aims of the study

1. To validate the use of a single clear fluid specimen passed per rectum during WGL as a representative of the rest of the WGL effluent in research into gut immunity.

2. To study gut immunity in heavy smokers and non-smokers, comparing levels of intestinal immunoglobulins and antigen-specific antibodies in both groups.

3. To investigate the intestinal immune responses to salmonella antigens, by using the newly licensed live oral typhoid vaccine Ty21a as an antigen, in a group of British healthy volunteers and also to examine the intestinal immune responses in a group of patients who had had natural infection with *Salmonella* spp.

4. To check and confirm the presence of a previously unrecognised condition, intestinal IgA deficiency. In fact, this aim arose in the course of my research

into gut immunity when I found several subjects who had absent or near absent IgA in the intestinal secretions but had normal levels of IgA in serum.

Approach

In the first instance protocols for a clinical study based on the use of whole gut lavage fluid were validated by establishing methods for collection and processing WGL specimens to be used as materials for quantitative investigations of intestinal immunoglobulins and specific antibodies both in healthy volunteers and patients with a variety of gastrointestinal diseases. A modified enzyme-linked immunosorbent assay (ELISA) technique was developed for the isotype-specific measurement of anti-*Salmonella typhi* LPS antibodies concentrations applicable to gut lavage fluids and serum. A standard (ELISA) technique was employed for the quantitative study of immunoglobulin concentrations in WGL fluid. A blood sample was collected from one of the gastrointestinal laboratory staff three weeks after she had TAB vaccine (parenteral vaccine against typhoid, paratyphoid A and paratyphoid B infection) and she was found to have a high titre of specific anti-*Salmonella typhi* LPS antibodies in serum. The serum then was divided into small aliquots and stored at -70 °C to be used as a standard for the isotype-specific ELISA. The antibodies of interest in these assays were those from a group of patients who had had naturally-acquired salmonella infection and a group of British adult healthy volunteers who had the oral typhoid vaccine Ty21a.

Following these technical developments, I started my clinical research by investigating 4-6 serial lavage samples from patients with a variety of gastrointestinal diseases to examine the validity of using the first clear sample as a representative for the gut lavage output. Later on, to study the influence of heavy smoking and non-smoking on the gut immunity, a group of young healthy volunteers were recruited from the computerised data of the Clinical Research Centre of the University of Edinburgh and were divided into heavy smokers and non-smokers. A number of gastrointestinal laboratory staff and their relatives who wished to participate in this study were also recruited.

To study intestinal immunity following a naturally-acquired salmonella infection and the duration of the intestinal immune response, a group of patients who had had *Salmonella typhi* or non-typhoidal salmonella infection (salmonellosis) in the preceding twelve months and who were admitted, treated, discharged and wished to take part in my study were referred from the City Hospital. I then examined levels of specific antibodies to the oral typhoid vaccine Ty21a and compared these levels with the levels of specific antibodies in the naturally-acquired salmonella infection as well as in patients with up-regulated intestinal humoral immunity (inflammatory bowel disease).

Finally, preliminary clinical and immunological investigations were performed in a group of patients who were found to have very low or no IgA at all in their lavage fluids but to have normal levels of IgA in serum. Immunological investigations

were extended to previously collected, processed and stored WGL fluids during the years 1991 and 1992 screening for intestinal IgA deficient patients. A blind microscopic examination of a mixture of intestinal biopsies from patients with intestinal IgA deficiency and a group of patients with a variety of gastrointestinal diseases (used as controls) was undertaken screening for IgA-producing plasma cells.

Chapter II

REVIEW OF THE LITERATURE

Section 1

THE GUT AS AN IMMUNE ORGAN

Introduction

The interaction between host and external environment occurs along two major surfaces, the skin and the mucosal surface. The mucosal surface involves an area much larger than the area covered by the skin (the surface area of the small intestine is about 300-400 m^2 compared with 1.8 m^2 for the skin). The intestinal immune system is separate from the systemic immune system and the gut is regarded as the largest immune organ in the body. Furthermore, the intestine is regarded as the largest immunoglobulins and antibodies forming organ in the human body and plays a protective role in the host defence against constant challenge by a wide spectrum of potentially harmful antigens.

The clinical importance of the mucosal defence system can be appreciated if one were to assess the implication of the infectious diarrhoea world-wide. Around 750 million episodes of diarrhoea caused by infectious agents occur every year among children below the age of 5 years in Asia (excluding China), Africa and Latin America and 5-10 million children world-wide die every year because of diarrhoeal diseases (World Health Organisation, 1984). The wide spectrum of gastrointestinal infections among patients with the Acquired Immunodeficiency Syndrome (AIDS) is another example of the important role

played by the mucosal defence system in healthy individuals. Therefore, an understanding of the mechanisms of the intestinal immune defence system will have a major implication for the prevention of diarrhoeal diseases e.g. by vaccination.

Historical review

The presence of a local intestinal immune system independent from the systemic immune system is not a new concept. Besredka (1919) reported that rabbits were protected against dysentery following orally administered killed *Shigella* bacillus, regardless of the immune response in the serum. Later on, Davis (1922) reported the presence of locally produced antibodies against the dysentery bacillus in the stools of infected persons before such antibodies appeared in serum. However, the interest in the intestinal mucosal immune system significantly increased only late in the 1960s after the discovery by Tomasi and Zigelbaum (1963) of IgA as a predominant immunoglobulin in the external secretions such as saliva, colostrum and lacrimal secretions. Locally produced IgA in saliva and colostrum was described by Hanson (1961), Tomasi et al (1965) and Chodirker et al (1963) in more detail. Secretory IgA (SIgA) was found to be different from serum monomeric IgA and to have unique chemical and immunological properties because of its dimeric character as well as its association with a secretory component (SC), which is an epithelial

glycoprotein. Tomasi et al (1965) had postulated that the SC is involved in the transport of IgA (and IgM) across the mucosal membrane into the gut lumen via a process called **transcytosis**. Accordingly, it was postulated that the dimeric SIgA is not transudated from serum but locally synthesised. This was shown definitively by Brandtzaeg (1974) who established a common epithelial transport model for dimeric IgA and pentameric IgM. Another important discovery by Crabbe et al (1965) has made a major contribution into the knowledge of the mucosal immune system. They demonstrated that the intestinal immunoglobulin-producing cells, unlike the other lymphoid tissue immunoglobulin-producing cells, were predominantly IgA-producing cells and that IgA-producing cells are 20 times as numerous as IgG-producing cells in the intestinal mucosa.

During the last two decades intensive studies into the regulatory mechanisms of the intestinal immune response have been carried out. The molecular and cellular interactions that regulate maturation and differentiation of lymphocytes into IgA-producing cells have been studied in animals (Clough et al, 1971; Torrigiani, 1972; Guy-Grand et al, 1975) and humans (Mestecky and McGhee, 1987; Conley and Delacroix, 1987; Brandtzaeg et al, 1985). However, the mechanism by which an intestinal immune response is established and regulated is, as yet, not well-understood.

Basic concept of gut immunity

The gut provides an important immunological barrier, in addition to its physiological function as the site of absorption of dietary nutrients, against a constant challenge by infectious, toxic and immunogenic materials. Potentially harmful antigens such as bacteria, viruses, protozoa and helminths are selectively excluded from entering into the systemic circulation by a process known as "Immunological Exclusion".

Immunological and non-immunological mechanisms of the mucosal defence system are involved in the defence against infectious agents. The non-immunological mechanisms include the following. (a) Intraluminal factors: such as gastric acid, bile salts, pancreatic proteases, and lysozymes; (b) Mucosal surface: these include mucous coat, enterocyte membrane integrity and epithelial tight junctions (Russell and Walker, 1990); (c) Normal bacterial flora: gut commensals produce endogenous antibiotics that are directly harmful to particular pathogens (MacKowiak, 1982); (d) Intestinal peristalsis.

The immunological defence is produced by the gut-associated lymphoid tissue (GALT) and involves the secretory immunoglobulins, mostly SIgA as the hallmark of the mucosal immune system and to a lesser extent IgM. The mucosa-associated lymphoid tissue (MALT) consists of the gut-associated, bronchus-associated and duct-associated lymphoid tissues and collectively

represent the largest lymphoid organ in the human body (Tomasi et al, 1980; Bienenstock, 1984; Bienenstock and Befus, 1984). GALT is composed of lymphoid aggregates, in Peyer's patches (PPs) and in the solitary lymphoid follicles (scattered throughout the gastrointestinal tract including the appendix) and lymphocyte populations in the gut epithelium (Intraepithelial Lymphocytes; IELs) and in the lamina propria (Trepel, 1974; Brandtzaeg, 1985; Owen, 1977).

Peyer's Patches (PPs)

In humans, PPs are well developed early in foetal life and at birth there are about 100 PPs increasing to about 250 PPs at the mid-teens then decreasing to about 100 PPs between the age of 70 and 95 years (Cornes, 1965). However, it is only shortly after birth that germinal centres appear in PPs (Bridges et al, 1959), which indicates the dependence of germinal centre formation on antigenic stimulation. PPs may be anatomically and functionally divided into three areas; the dome, the follicles and the interfollicular areas. The dome is covered by an epithelium containing specialised cells called "M" (for microfold) cells. M cells are involved in the transport of antigens inward across the PPs epithelium via a process known as **pinocytosis**. M cells are thin cells (the thickness of the cytoplasm is only 0.1-0.3 μm) and the underlying lymphocytes are as close as 0.3 μm from the gut lumen (Guy-Grand et al,

1975; Owen, 1977; Bockman and Cooper, 1975). M cells are described below in more detail.

The follicles, or B-cells zones, with germinal centres contain 60-70% of the membrane-bound IgA positive ($mIgA^+$) B cells. These cells are regarded as the immediate precursor of IgA-producing plasma cells and the PPs are thought to be the site where the initial commitment of B cells to produce IgA is made (Butcher et al, 1982). Two types of $mIgA^+$ B cells are found in PP germinal centres; small resting memory B cells and large dividing B cells (Butcher et al, 1982; Lebman et al, 1987). The final differentiation of B cells into IgA-producing plasma cells, however, does not occur in B cells zone of PPs but in the distant effector sites of the gut mucosa.

The third area in PPs is the parafollicular (or interfollicular) area, or T cell area (McGhee et al, 1989). The majority of T cells in the parafollicular area are $CD3^+$, $CD4^+$, $CD8^-$, and $CD5^+$ (Guy-Grand et al, 1975). These are known to be inducer cells or T helper (Th) cells. However, a significant number of $CD4^-$, $CD8^+$ suppressor/cytotoxic T cells are also found in PPs (Guy-Grand et al, 1975). The presence of large numbers of Th cells in PPs supports the suggestion that PPs are the site of induction of the intestinal immune response. Morphological and functional studies of PPs have revealed the presence of unique T lymphocytes and dendritic cells that regulate the differentiation of B cells and influence their maturation and immunoglobulin

isotype switching to predominantly IgA-producing B cells (McGhee et al, 1989).

Intestinal M Cells

Kumagai (1923) demonstrated that there was selective uptake of dead mycobacteria from the lumen of the gut into PPs and the appendiceal lymphoid tissues. However, the distinctive description of M cells was only made by Schmedtje more than 40 years later when he described them as "lymphoepithelial cells" overlying the lymphoid nodules (Schmedtje, 1965). In humans, about 10% of PPs dome epithelial cells are M cells while in rabbits M cells constitute up to 50% of the dome epithelium (Trier, 1991).

Under electron microscopy, human M cells appear as short, irregular networks of folds that strikingly contrast the regular carpet-like appearance of the microvilli on adjacent absorptive cells (Owen and Jones, 1974). The apical plasma membrane of M cells differs considerably from that of the adjacent absorptive cells; for example it is selectively adherent for reovirus type 1, some strains of *E. coli* and cholera toxin B-subunit, and facilitates their transepithelial transport (Trier, 1991). Furthermore, luminal SIgA and some lectins were found to bind selectively to the apical membrane of M cells (Trier,

1991). This unique structure of M cells has led to the concept that M cells are the site of antigen sampling.

The passage of intact molecules across the gut is at variance with the role of the gut as a molecular barrier. However, macromolecules and micro-organisms that survive intraluminal degradation have the potential to adhere selectively to the apical surface of M cells then be endocytosed into M cells or transported across the cytoplasm of M cells crossing to the underlying immunocomponent cells of PPs. M cells are involved in transepithelial transport of macromolecules that adhere selectively to the apical surface of M cells, such as cholera toxin B-subunit and certain lectins, by adsorptive endocytosis whereas molecules that do not adhere to the apical surface of M cells, such as albumin, are transported by a fluid phase endocytosis (Neutra et al, 1987). Micro-organisms such as some viruses (Sicinski et al, 1990), bacteria (Owen et al, 1986) and even protozoa, for example *Cryptosporidium* may also adhere selectively to the apical surface of M cells and be transported to the underlying lymphoid cells of PPs (Marcial and Madara, 1986). The interaction of bacteria with M cells seems to be influenced by the virulence of the organism as well as the host immune factors. Most of the commensal intestinal bacteria, which constitute the normal gut flora do not generally adhere to the apical surface of M cells of healthy mammals whose intestinal flora has not been disturbed (Trier, 1991). Pathogens such as the RDEC-1 strain of *E. coli*, an enteroadherent organism that produces diarrhoea in rabbits without invasion or

production of classic enterotoxins, and *V. Cholerae* may adhere selectively to or invade M cells with minimal damage (Inman and Cantey, 1983; Owen et al, 1986). Other virulent pathogens such as *Salmonella typhi* and *Shigella flexneri* use M cells as a portal of entry into the systemic circulation and produce extensive dome ulceration with destruction of the PPs epithelium (Kohbata et al, 1986; Wassef et al, 1989).

The regulatory mechanism by which macromolecules and micro-organisms either selectively adhere to or invade M cells, and the mechanisms by which these macromolecules and micro-organisms are taken up into or transported across the cytoplasm of M cells are not, as yet, well understood. Studies in rabbit (Kato, 1990) and mouse (Roy and Varvayanis, 1987) showed that there is selective adherence of intraluminal SIgA to M cells of these animals. In another study, the delivery of reovirus type 1 specific monoclonal IgA antibodies into the intestinal lumen of mice resulted in marked inhibition of viral entry to or replication in the mucosa following oral challenge with reovirus type 1 (Weltzin et al, 1989). One interpretation of the above observations is that the selective adherence of specific IgA antibodies to M cells may inhibit rather than facilitate the uptake of micro-organisms or antigenic macromolecules to which specific IgA antibodies are directed. This would prevent enteric and systemic infections and modulate potentially damaging immune responses.

Secretory IgA (SIgA)

The secretion of SIgA into external body fluids, including those of hepatobiliary and gastrointestinal tract, is the hallmark of the mucosal immune system. In humans, the combined daily synthesis of systemic and SIgA was estimated as 66 mg/kg body weight per day of which 40 mg/kg body weight per day is the SIgA that is secreted into the lumen of gut in adults (Conley and Delacroix, 1987), i.e. approximately 3g of SIgA is transported into the gut lumen of the average adult per day. The total daily production of IgG, IgM, IgD and IgE was estimated as 34, 8, 0.4 and 0.02 mg per kg body weight respectively (Conley and Delacroix, 1987). Therefore, the overall daily production of SIgA is larger than all other isotypes combined. In humans, IgA-producing cells in the systemic and secretory compartments markedly outnumber those secreting other isotypes. Enumeration of immunoglobulin-producing cells in various tissues showed that 1-2 m of human intestine contained more immunoglobulin-producing cells (predominantly of the IgA isotype) than did all other tissues combined (Brandtzaeg and Baklien, 1976; Brandtzaeg et al, 1991). Since the majority of infectious agents are encountered in the lumen and mucous membrane of the gut, then the need for synthesis and secretion of such a large amount of IgA is obvious.

Structure of SIgA. IgA is found in two different molecular forms, monomeric (mIgA) and polymeric (pIgA) (Mestecky and McGhee, 1987; Conley and

Delacroix, 1987; Mestecky et al, 1986). In humans, immunochemical and biochemical studies showed that there are two subclasses of IgA: IgA1 and IgA2 and that these two subclasses have different distribution in various body fluids (Mestecky and McGhee, 1987; Mestecky and Russell, 1986; Delacroix et al, 1982). In humans, 85-99% of IgA in serum is in the monomeric 7S form (Mestecky and Russell, 1986) and up to 85% of IgA in serum is IgA1 subclass. SIgA, on the other hand, is mainly found in 11S polymeric form, usually as dimers. However, trimers, tetramers and pentamers are produced in lesser amount (Mestecky et al, 1986). While serum IgA is predominantly monomeric IgA1, in external secretions, IgA is predominantly polymeric and up to 95% of IgA in gastrointestinal secretions is secretory IgA with an increased proportion of IgA2 subclass (IgA1:IgA2 is 70:30 in gastrointestinal secretions compared with 85:15 in serum) (Delacroix et al, 1982; Kerr, 1990).

The distinctive feature of IgA is its ability to form polymers by having special heavy chain constant-region (CH) terminal extensions with an extra cysteine residue which can participate in cross-linking monomeric subunits together via disulfide bonds (Mestecky and McGhee, 1987). The typical monomeric IgA molecule is composed of two α heavy and two light chains (either κ or λ) linked by covalent (disulfide) and non-covalent bonds (Mestecky and McGhee, 1987; Mestecky et al, 1986). The typical dimeric molecule of SIgA , on the other hand, is composed of two monomeric IgA subunits linked together by disulfide bonds, one Secretory Component (SC) and one J (joining) chain

linked to the Fc region of the polymers (Halpern and Koshland, 1973; Koshland, 1985; Mestecky and McGhee, 1987). J chain is acquired during the process of polymerisation in plasma cells while SC is acquired during the selective transport of SIgA through the epithelial cells (Mestecky et al, 1991).

J chain is a glycoprotein that is found in polymeric IgA and IgM of many species. It is produced in B cells and in plasma cells during the various stages of differentiation of these cells. The function of J chain has been found not only to link IgA monomers together via disulfide bonds (Garcia-Pardo et al, 1981; Brandtzaeg and Prydz, 1984) but also to enable pIgA to bind to SC although SC does not bind directly to the J chain (Eiffert et al, 1984). Polymeric IgA containing J chain has been shown to have an increased resistance to proteolytic enzymes (Koshland, 1985).

The most important feature of SIgA is the presence of an associated glycoprotein piece called the secretory component (SC). SC is responsible for the selective transport of pIgA across the epithelial cells. It also has been found to stabilise the quaternary structure of IgA and increase its resistance to the proteolytic enzymes (Brown et al, 1976). SC is found in three molecular forms (Mestecky et al, 1991): (a) as a membrane protein on the basolateral surface of the epithelial cells where it functions as a receptor for polymeric immunoglobulins (polymeric immunoglobulin receptor; pIgR), (b) as a

component of polymeric immunoglobulins and (c) as a free glycoprotein found in many external secretions. The molecular structure of SC is composed of a single polypeptide chain with 549-558 amino acids and large amounts of carbohydrates folded into five domains (homology regions) each comprises 104-114 amino acid residues and contains intrachain disulfide bridges (Eiffert, 1984). The fifth domain contains an additional intrachain disulfide bond which is involved in the disulfide bridges that connects SC to the α chains of one of the monomers in dimeric IgA. PIgR is synthesised and expressed on the surface of epithelial cells of the gastrointestinal and respiratory tracts, acinar and ductal cells in exocrine tissues as well as the lining cells of uterus (Mestecky et al, 1986; Brandtzaeg, 1985). Polymeric IgA with J chain binds to the pIgR at the basolateral surface of the epithelial cells, is transported across the cytoplasm of epithelial cells via endocytotic vesicles and then secreted at the epithelial surface as SIgA (Brandtzaeg and Baklien, 1976). Part of the receptor cleaves off and remains attached to the pIg and is known as SC. Because cells involved in producing SC and transporting SIgA are constitutively synthesising and secreting SC, thus SC can be found as free SC in the secretions (Brown et al, 1976).

Source of Intestinal IgA. In serum, the bone marrow has been regarded as a major source of IgA in mice (Benner et al, 1981) as well as in humans (Conley and Delacroix, 1987). It was postulated that mature B cells leave the bone

marrow to become activated following interaction with T cells and antigen-presenting cells in the peripheral lymphoid organs, then return to the bone marrow where they differentiate into immunoglobulin-producing plasma cells (Benner et al, 1981). On the other hand, GALT, represented by PPs, have been regarded as the source of IgA-producing plasma cells found in distant mucosal tissues including the intestine, mammary, lacrimal, salivary and uterine glands as well as the respiratory tract (Mestecky and McGhee, 1987). During early ontogeny, B and T lymphocytes precursors, presumably of bone marrow origin, migrate to PPs, via interfollicular postcapillary venules (Mestecky et al, 1991; Brandtzaeg et al, 1991). Thereafter, preferential compartmentalisation occurs.

Function of SIgA. The function of SIgA is mainly to provide an immunological barrier against a variety of foreign antigens including food antigens, bacteria, viruses, parasites, and toxins. Possible mechanisms by which SIgA may protect against enteric infection are described below. (a) direct action by immobilisation, agglutination and prevention of bacterial adherence to the mucous membrane and (b) combination with bacterial products such as toxins or enzyme resulting in inactivation and destruction of these products by the proteolytic enzymes of the intestine (World Health Organisation, 1979; Bienenstock and Befus, 1985).

Attachment of micro-organisms to epithelial cells is thought to be mediated by receptors such as GM1 on the epithelial cells (to which cholera toxins bind) and specific attachment sites on the pathogens such as fimbria and pili (Tse and Chadee, 1991). Adherence of micro-organisms to the epithelial cells may be prevented by coating of the micro-organisms with SIgA resulting in blocking of the sites of attachment retaining the micro-organisms within the mucous layer (Childers et al, 1989). Once the mobility of the pathogens is reduced, they are susceptible to proteolytic enzymes and may be easily swept out by peristalsis.

In animals, studies have shown that intestinal loops of orally immunised mice were partially protected by specific IgA against live *Vibrio Cholerae* (Fubara and Freter, 1973). Similar observation has been made in studies on *E. coli* (strain RDEC-1) in a rabbit model, in which specific IgA antibodies in milk protected the animal from diarrhoea (Boedeker et al, 1987). In the case of *Salmonella*, SIgA attached to the organisms may prevent their adherence to the intestine by causing a decrease in hydrophobicity and in negative charges of the organisms (Magnusson et al, 1979; Magnusson and Stendhal, 1982).

In humans, among the most convincing results of studies on the antimicrobial function of SIgA is the work of Ogra et al (1968) who showed that oral immunisation with live poliovirus vaccine, but not with killed poliovirus vaccines, produced an intestinal immune response with the production of SIgA

antibodies. The intestinal immune response was associated with significant protection against poliovirus. In clinical studies, oral cholera vaccine, consisting of the nontoxic but highly immunogenic B subunit of cholera toxin together with heat- and formalin-killed whole cell *V. Cholerae* organisms (B-WC), has been found to stimulate gut mucosal anti-toxin and anti-bacterial IgA (Svennerholm et al, 1984) and administration of this vaccine has been associated with long-term protection against *V. Cholerae* (Clemens et al, 1986; Clemens et al, 1990).

The role of SIgA in excluding potentially dangerous immunogens is not limited to the prevention of bacterial antigens from reaching the systemic circulation but also plays an important role in limiting uptake of potentially harmful food antigens across the intestinal epithelium. SIgA, together with some food antigens, form immune complexes that can not bind to the surface of absorptive cells (Walker et al, 1975). In patients with selective IgA deficiency and new-born infants with transient IgA deficiency, high titres of antibodies against food antigens can be detected in their serum (Cunningham-Rundles et al, 1978; Eastham et al, 1978).

In some circumstances, ingestion of an antigen may lead to oral tolerance rather than induction of intestinal immune response. Oral tolerance is described as an antigen-specific hyporesponsiveness of systemic humoral or cellular

immunity induced by antigen feeding prior to systemic challenge (Bruce and Elson, 1990). Absence of oral tolerance may result in autoimmune diseases such as autoimmune enteropathy and pernicious anaemia (Roitt et al, 1965), coeliac disease and cow's milk enteropathy (Bruce and Elson, 1990; Mirakian et al, 1986). Recovery from cow's milk enteropathy was recently found to be positively correlated with the presence of intestinal SIgA against milk proteins (Isolauri et al, 1992). Thus, ingestion of an antigen may (a) induce oral tolerance (b) initiate a local immune response; and (c) (very rarely) may result in inappropriate systemic immune responses (Sanderson and Walker, 1993).

As cited above, prevention of potentially harmful antigens, by SIgA, from reaching the systemic circulation has been suggested to occur through the "immune exclusion" mechanism. Therefore, it seems that the protective function of SIgA is not limited to the mucosal surface but it may function within the mucosa itself, i.e. in the lining epithelium and the lamina propria, to prevent pathogens that succeeded in gaining access to the mucosa from reaching the systemic circulation. In a study *in vitro* (Mazanec et al, 1993), virus titres were reduced when monoclonal IgA antibodies specific for viral envelope proteins were added to an epithelial model system, polarised monolayer cells of Madin Darby canine kidney (MDCK), infected with *Sendai* or *influenza* viruses suggesting intracellular viral neutralisation by specific IgA. Furthermore, antigens that have been absorbed intact across the mucosal

epithelium may form immune complexes with locally produced IgA antibodies in the lamina propria (Husby et al, 1985). In addition, Mazanec and colleagues (Mazanec et al, 1993) have suggested an excretory function for SIgA. They demonstrated, in a study *in vitro*, that immune complexes containing dimeric IgA, but not monomeric IgA or IgG antibodies, were readily transported across the epithelial cells into the apical medium (representing the external surface). Therefore, it appears that polymeric IgA is involved in an efficient local removal of potentially dangerous antigens in the form of immune complexes from the mucosa, thereby preventing harmful systemic immune reactions.

Induction and expression of mucosal immune responses

Mucosal immune response can be divided into **afferent** (induction) and **efferent** (effector) phases. The afferent phase of the mucosal immune response starts by selective transport of antigens by M cells into PPs. Antigens are then taken up by antigen-presenting cells (APCs) (accessory cells) such as macrophages and dendritic cells. Antigens are taken up by APCs via a process called **endocytosis** then degraded, by a serious of degradative enzymes present in the endocytotic vesicle, into small antigenically distinct peptides (Brandtzaeg et al, 1989). These peptides interact with class II Major Histocompatibility Complexes (MHC) antigens to form complexes which are then re-expressed on

the cell surface. Only cells expressing class II MHC molecules can serve as antigen-presenting cells (Brandtzaeg et al, 1989; Mayer, 1990). Class II MHC molecules are found on macrophages, dendritic cells, B-cells, activated T-cells and, in some circumstances, on intestinal epithelial cells (Elson et al, 1986). Processed antigens bound to class II MHC molecules on the surface of APCs can be recognised by the receptor of specific helper T cells (Th) which then become activated and secrete lymphokines such as IL-2, IFN-γ, B-cell growth factors and differentiation factors (Mayer, 1990).

Activated APCs synthesise and release IL-1, which is an important factor mediating the interaction between APCs and T cells contributing to activation of T cells and the release of IL-2 (Elson et al, 1986; James, 1991). Activated T cells release factors which act on IgM-bearing B cells ($mIgM^+$) to switch to IgA-bearing B cells ($mIgA^+$) (Kawanishi et al, 1983a; Kawanishi et al, 1983b).

Live micro-organisms, as particulate antigens, are processed by macrophages and dendritic cells in PPs resulting in an efficient mucosal immune response whereas killed micro-organisms, as soluble antigens after being degraded by proteolytic enzymes and transformed into small peptides, are processed by macrophages present in lamina propria of the villi rather than in PPs resulting in tolerance rather than immune response (Biewenga et al, 1993; Pavli et al, 1990). Despite the fact that dendritic cells are non-phagocytic cells and antigen

processing occurs outside the cell, yet they are regarded as the most potent APCs (Steinman and Cohn, 1973). This may be due to the fact that dendritic cells are rich in class II MHC molecules and have strong avidity to T cells (Nussenzweig and Steinman, 1980). Dendritic cells have been found to constitute the main APC population in the dome area of PPs (Biewenga et al, 1993). However, dendritic cells cannot work independently and can only work as a part of the accessory cells network (Witmer-Pack et al, 1987).

Factors influencing IgA B cells differentiation

There is an increasing evidence that the immunoglobulin isotype switch of B cells to IgA-producing cells is a directed process under the control of cellular and soluble factors called lymphokines. Kawanishi et al (1983) have suggested that the presence of activated T cells is necessary for the isotype switch to IgA production in murine B cells. Further studies by Spalding and Griffin (1986) showed that dendritic cells derived from PPs stimulated pre-B cells to switch to IgA-producing B cells. Furthermore, "stromal" cells may be involved in the induction of $sIgM^+$ B cells to switch to $sIgA^+$ B cells (Strober and Harriman, 1991). Although the nature of the cell involved in IgA B cells switching is not known, the above studies suggest that there must be a cell within PPs that is involved in IgA B cell switching.

Other studies showed that lymphokines are also involved in the process of IgA B cell switching and differentiation. IL-4 was suggested to be an important factor that, in certain circumstances, together with transforming growth factor-β (TGF-β) support the early switching of $mIgM^+$ to $mIgA^+$ B cells (Strober and Harriman, 1991; Biewenga et al, 1993). Regulation of secretion of IL-4 and other co-factors involved in isotype switching and differentiation of B cells may be controlled by helper T cells. In this model, two types of helper T cells were initially described, Th1 and Th2 (Cherwinski et al, 1987). Th1 cells have been suggested to produce IL-2 and INF-γ while Th2 cells produce IL-4, IL-5 and IL-6. While Th2 cells promote the production of IgA, IFN-γ from Th1 cells acts on Th2 cells resulting in suppression of IgA production (Biewenga et al, 1993). However, the precise mechanism that controls activation and inhibition of Th1 and Th2 is not, as yet, well-understood.

Terminal differentiation of IgA-producing B cells (post-switch step) into IgA-secreting plasma cells occurs in the lamina propria but not in PPs. Therefore, stimulated (switched) $mIgA^+$ B cells migrate to the mesentric lymph nodes, to the thoracic duct, and then to the systemic circulation. From the systemic circulation, stimulated $mIgA^+$ B cells selectively migrate to the effector sites of the mucosal immune response i.e. the lamina propria. Lymphocyte "homing" is a process in which IgA committed B cells migrate to the organ where antigenic stimulation initially occurred. Once there, the lymphocytes differentiate into

antibody-secreting plasma cells and die within a few days (Husband and Gowans, 1978).

Differentiation of $mIgA^+$ B cells in the lamina propria requires the presence of IL-5 while the final differentiation and maturation of activated B cells to IgA plasma cells require the presence of IL-6 and IL-2 together (Strober and Harriman, 1991).

Finally, better understanding of the mechanisms regulating IgA induction and secretion may help not only in developing methods for protection against mucosal pathogens but also in the management of immunological disorders that may be caused by mucosal immune dysfunction.

Section 2

INVESTIGATION OF THE INTESTINAL IMMUNE SYSTEM

Introduction

Traditional methods for studying systemic immune responses depend heavily on detection of antibodies in serum while study of human intestinal immunity has been hampered by the difficulty of obtaining access into the intestinal secretions. Instead, studies of the intestinal immune system have frequently relied on indirect methods for investigation of the intestinal immune responses to antigens such as determination of levels of antigen-specific antibodies in serum (Levine et al, 1989; Levine et al, 1988; Megasena et al, 1989), saliva (Jertborn et al, 1984) and colostrum (Jertborn et al, 1986) or peripheral blood lymphocytes (Czerkinsky et al, 1987; Kantele, 1990; Kantele et al, 1986; Forrest, 1992a). However, it is now widely accepted that the intestinal immune system is largely independent from the systemic immune system and the findings in serum do not necessarily reflect the immunity at the intestinal levels. Therefore, development of direct methods for investigation of the mucosal immune system has become necessary.

Obtaining intestinal fluids in which to study the intestinal immune system has been regarded as the "gold standard" by which other methods may be compared. Therefore, as a direct approach to the intestinal fluids, saline lavage was used

(Svennerholm et al, 1984; Svennerholm et al, 1982). However, saline lavage may be contraindicated in certain groups of patients such as cardiac, renal and liver disease patients. Another method of direct approach to the intestinal fluids is intestinal intubation with direct sampling of jejunal fluids (Forrest et al, 1991; Forrest et al, 1992; LaBrooy et al, 1980; LaBrooy et al, 1982). However, intestinal intubation is an invasive and inconvenient technique for the patient as well as for the investigator. Furthermore, intestinal intubation is a labour-intensive and time-consuming procedure.

The use of faecal extracts to study gut immunity

In an attempt to overcome the difficulty of obtaining direct access into the intestinal secretions, and because of the lack of other reliable methods in the past, some workers used faecal extracts to study the intestinal immune responses to natural infections or to orally administered antigens (El-Hawy et al, 1983; Reed and Williams, 1971; Northrup et al, 1970; Plaut and Keonil, 1969; Chodirker and Tomasi, 1963). However, the damaging effects of the proteolytic enzymes in the intestinal secretions on the immunoglobulins and specific antibodies in faecal extracts resulted in misleading results. Furthermore, interpretation of the results of measurement of immunoglobulins and antibodies in the faecal extracts depends on the faecal weight, and because of the variation in water contents of faecal specimens, there have been problems in the interpretation of results based on faecal

samples. O'Mahony and colleagues (1990) reported that analysis of semi-liquid early faecal specimens obtained during the process of whole gut lavage (WGL) contained negligible quantities of IgA compared with the amount of IgA found in clear specimens of WGL fluid. Later on, studies in the same centre (Ferguson et al, 1994) of paired samples of faeces and whole gut lavage fluids from ten patients with a variety of gastrointestinal diseases showed that specimens of WGL fluids contained much higher quantities of IgA than did the specimens of faecal extracts.

The use of whole gut lavage to study gut immunity

Elson and colleagues (1984) described the use of whole gut lavage (WGL) as a non-invasive method for the quantitative measurement of immunoglobulins and antigen-specific antibodies in mice. They carried out WGL using a solution containing (162g/l) polyethylene glycol with an average molecular weight of 3350 plus traces of electrolytes such as sodium and potassium. Later on, Gaspari and colleagues (1988) described a modified WGL technique using commercially available polyethylene glycol (Golytely) in normal human volunteers. Golytely has essentially been used clinically for cleansing the colon as a preparation for Barium enema, colonoscopy and for surgical procedures in the colon. Golytely has been found not to produce net absorption or secretion of fluids or electrolytes across the mucosal barrier of the gut (Davis et al, 1980) and hence, can be used safely in patients with liver, renal or cardiac diseases (unlike saline lavage).

Human intestinal secretions contain large amount of protease enzymes which, if not inhibited, significantly reduce the amount of immunoglobulins in WGL fluids including SIgA despite the known resistance of SIgA to proteolytic enzymes (Brown et al, 1970; Hohmann et al, 1983; Samson et al, 1973; O'Mahony et al, 1990). The loss of immunoglobulin contents of WGL fluids due to proteolysis can be prevented by treating the lavage specimens with protease inhibitors such as soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (PMSF) as well as chelating agents such as ethylene diamine tetracetic acid (EDTA) (Elson et al, 1984; Gaspari et al, 1988; O'Mahony et al, 1990). Calf serum is added as an alternative substrate for any remaining proteases in WGL fluids (Elson et al, 1984; Gaspari et al, 1988). Adding protease inhibitors was found not only to decrease the loss of immunoglobulins in WGL fluids but also it to improve antigen stability and to increase the sensitivity of the enzyme-linked immunosorbent assay (ELISA) used to measure the amount of immunoglobulins and antigen-specific antibodies in intestinal fluids (Hohmann et al, 1983). Storage of intestinal fluids at -70 °C has been shown to maintain the immunoglobulin and specific antibodies content of these fluids for up to one year (Forrest, 1992b). Azacol (a commercially available reagent) has been used for the assessment of the activity of protease enzymes in the intestinal secretion obtained by the WGL method (Elson et al, 1984).

Radial immunodiffusion has been used to estimate IgA concentration in exocrine secretions, including intestinal secretions, but has been found to be relatively insensitive and gives inaccurate estimates of immunoglobulin contents of these

secretions due to the presence of different ratios of monomeric and polymeric forms of immunoglobulins in the secretions (Brown et al, 1970; Tomasi and Grey, 1972).

ELISA has been used for quantitative study of immunoglobulins and antigen-specific antibodies in the treated specimens of WGL fluids and has been shown to be sensitive and reliable in the study of total immunoglobulins and specific antibodies in exocrine secretions such as saliva, breast milk and intestinal fluids (Elson et al, 1984; Hohmann et al, 1983; O'Mahony et al, 1990; Sack et al, 1980; Svennerholm et al, 1977).

Applications of WGL in clinical research

In the GI Unit of the Western General Hospital, WGL, in addition to its use for cleansing the colon as a preparatory method for Barium enema and colonoscopy and also as a treatment for intractable constipation, has been used to investigate gut immunity in a variety of gastrointestinal diseases (O'Mahony et al, 1990; O'Mahony et al, 1991b; Brydon et al, 1993; Brydon and Ferguson, 1992; Choudari et al, 1993) as well as to investigate the relevance of host factors such as age and nutrition and their effect on gut immunity (Hodges et al, 1993). Fluids obtained by WGL technique normally contain traces of IgG, albumin, and alpha-1-antitrypsin (Brydon et al, 1993). WGL fluids obtained from patients with

inflammatory bowel diseases (IBD) were found to have higher concentrations of IgG and albumin than did normal controls (O'Mahony et al, 1991b; Choudari et al, 1993). Therefore, WGL technique can be used as an objective means of grading the loss of proteins and therefore disease activity in IBD patients.

Another use of WGL in clinical research is investigation of occult blood loss from the gastrointestinal tract (Brydon and Ferguson, 1992). In the above study, it was found that normal individuals may lose 0.1-1.1 ml of blood per day through their gastrointestinal tract but higher concentrations of haemoglobin were found in WGL fluids obtained from patients with colorectal cancer, rectal varices, severe diverticular disease and in some patients with active IBD.

WGL has also been used in children (6-9 years old) from Sierra Leone to study the relevance of age, infection, and malnutrition to intestinal immunity (Hodges et al, 1993). When compared with values for Scottish adults, WGL fluids from the Sierra Leonean children had significantly higher concentrations of total IgA and IgM as well as higher concentrations of IgA and IgM antibodies against dietary antigens and *Salmonella typhi* lipopolysaccharide (LPS).

The use of WGL technique in obtaining material to study the intestinal immune response to natural infection or to an orally administered vaccine is an important application of WGL in clinical research. In an initial study in this centre, WGL fluid was used to study the intestinal immune response to the live attenuated oral

typhoid vaccine Ty21a (Sallam and Ferguson, 1992). An extension of that preliminary work on the effect of the vaccine Ty21a on gut immunity forms an important component of this thesis and will be discussed later.

Section 3

BASIC STRUCTURE OF BACTERIA

Introduction

In 1866, Haeckel suggested that micro-organisms such as algae, protozoa, fungi and bacteria should be placed in a separate kingdom called the *protista*. However, in the middle of this century electron microscopy showed that bacteria have more primitive cellular structure than the other three groups. Bacteria are classified as **prokaryotes**, while algae, protozoa and fungi are classified as **eukaryotes**. The eukaryotes also include plants and animals (Carlile and Skehl, 1974). The main differences between prokaryotes and eukaryotes are summarised in Table 2.I.

features	prokaryotes	eukaryotes
Chromosome no.	single	multiple
Nuclear membrane	absent	present
Mitochondria	absent	present
Multiplication	by binary fission	by mitosis

Table 2.I. The main differences between prokaryotes and eukaryotes.

Prokaryotes are further classified into three main groups: Eubacteria, Archaebacteria, and Cyanobacteria. Cyanobacteria, though a true prokaryote (Laskin and Lechevalier, 1977; Laskin and Lechevalier, 1979), are chlorophyll-containing and hence, previously were called the blue-green algae. Archaebacteria have the most primitive cellular structure and include 3 groups of prokaryotes. These are the Methanogens, extreme Halophiles and Thermoacidophiles. The Eubacteria (all prokaryotes other than Archaebacteria and Cyanobacteria) can be classified into Bacteria, Rickettsia, Chlamydia and Mycoplasma. Rickettsia and Chlamydia differ from bacteria in being smaller and strictly intracellular parasites (Woese and Wolfe, 1985).

General structural features of bacteria

The cell envelope

The cell envelope in the prokaryotes has 2 main components: an inner layer called the **cytoplasmic membrane** and an outer layer called the **cell wall.** The structure of the cell envelope is different in Gram-positive from that of Gram-negative bacteria (Cheesbrough, 1984). The cell envelope in Gram-positive bacteria consists of 2 layers; the cytoplasmic membrane and a thick peptidoglycan layer (mucopeptide). An outer layer called the **capsule** is often present. The cell envelope in Gram-negative bacteria is more complex than in Gram-positive

bacteria. It consists of the cytoplasmic membrane (also called the inner membrane) surrounded by a thin sheet of peptidoglycan attached to a complex layer called the outer membrane, which contains the **lipopolysaccharide** (endotoxin) (Sleigh and Timbury, 1981). The outer membrane is often surrounded by an outmost layer called the capsule. The gel-filled region between the inner and outer membrane is called the **periplasm** (Fig. 2.I).

The cytoplasmic membrane

The cytoplasmic membrane is the site of many major cellular functions (Jawetz et al, 1987): (a) selective permeability and transport of solutions; (b) electron transport and oxidative phosphorylation; (c) secretion of extracellular hydrolytic enzymes (exoenzymes); (d) biosynthesis of phospholipids and proteins (e); chemotactic function.

Bacterial cell wall

Bacterial cells contain high concentrations of inorganic ions and thus have high osmotic pressure (5-20 atmospheres) (Hancock and Poxton, 1988). Therefore, bacterial cells need strong cell wall to resist swelling due to fluid influx into the cells causing their lysis. Furthermore, the cell wall maintains the shape of the cell

and protects the mechanically fragile cytoplasmic membrane. Bacterial cell walls are described in more detail later in this chapter.

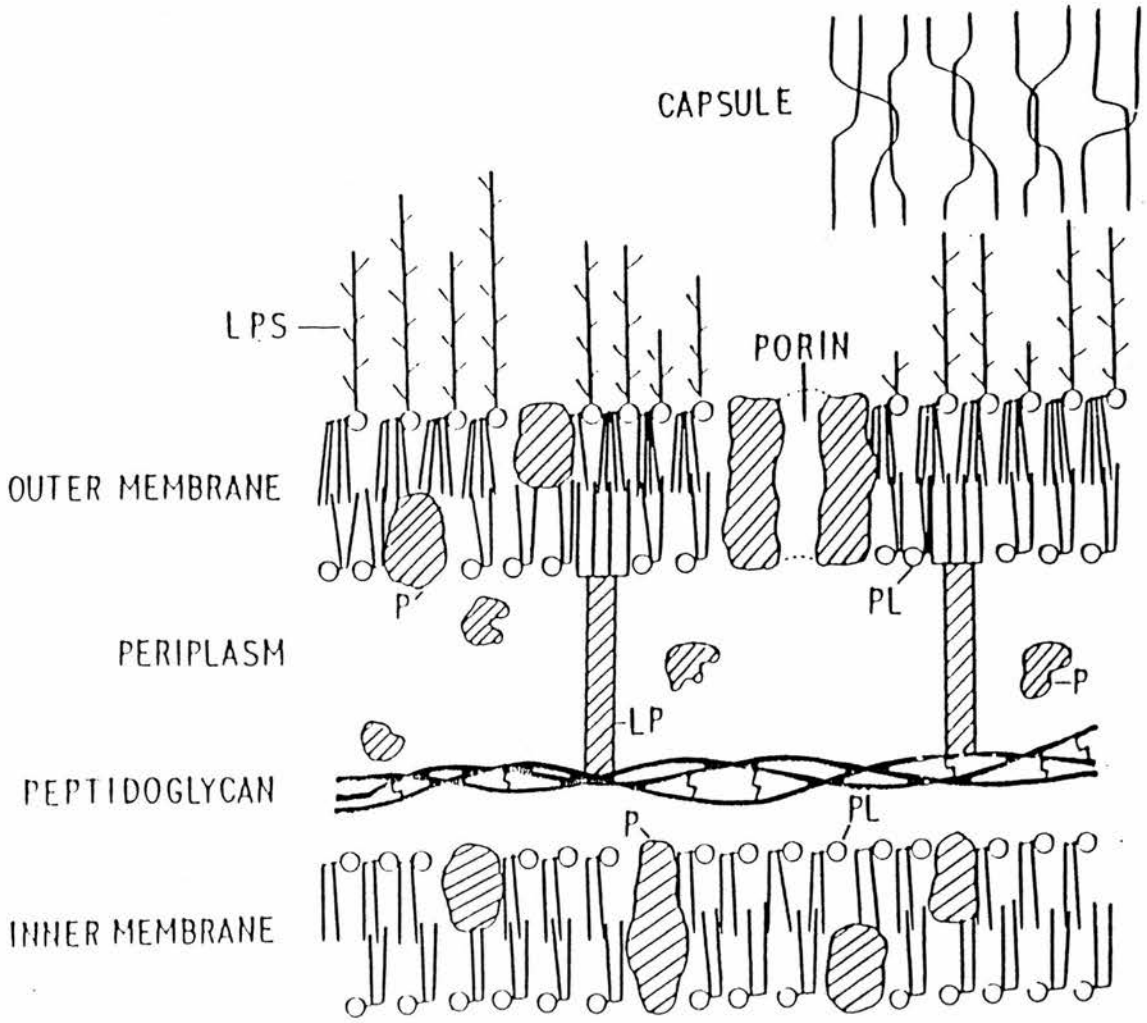


Fig. 2.I The cell envelope of a Gram-negative bacterium. LP= lipoprotein; LPS= lipopolysaccharide; P= protein; PL= phospholipid. (From Hancock and Poxton, 1988.)

Bacterial capsule

Many bacteria are surrounded with a large amount of extracellular polysaccharide. This polysaccharide layer is also known as the 'Slime layer'. When the polysaccharide layer is condensed and well-defined, it is called the capsule. However, when this layer is a loose meshwork, it is called the glycocalyx. The presence of the capsule is often correlated with the virulence of the pathogenic bacteria. Encapsulated bacteria are protected from being phagocytosed. Anti-capsular antibodies, however, can counteract the presence of the capsule (Jawetz et al, 1987).

The nucleus

Bacteria lack true nuclei since there is no nuclear membrane or mitotic apparatus as found in eukaryotic cells. Under electron microscopy, the cytoplasm can be seen to be filled with DNA fibrils. The DNA of the bacterial nucleus is a single piece of double stranded DNA and is thus regarded as a single chromosome of about 1 mm long and 2-3 X 10^9 molecular weight. The nuclear DNA is associated with small amount of RNA and RNA polymerase. The DNA is folded around an RNA core which seems to keep the DNA in its condensed form (Pettijohn, 1976; Kleppe et al, 1979).

Ribosomes

These are distributed throughout the cytoplasm and are the site of protein synthesis. The cytoplasmic RNA of bacterial cells is of three types regarding function: ribosomal (rRNA), amino acid transfer or transfer RNA (tRNA) and messenger RNA (mRNA). Bacterial ribosomes have a sedimentation constant of 70S (compared to the human and eukaryotic ribosomes which are composed of 80S) (Svedberg unit, i.e. $S=10^{-13}$ cm/sec/unit field). The aggregates of the 70S particles are attached to a common strand of mRNA to form **polysomes** where the mRNA functions as the template for protein synthesis i.e. determines the order of amino acids in the peptide chain. In spite of the presence of many similarities between bacterial ribosomes and human cellular tissues, there are still some differences which allow the use of antibacterial agents such as streptomycin which interfere with bacterial metabolism at the ribosomal level without unduly affecting the human ribosomal function.

Inclusion granules

In many species of bacteria, round granules may be seen in the cytoplasm. These are known as inclusion granules. They are not essential structures and may be absent under certain condition of growth. Inclusion granules include the following (Shively, 1974). (a) Volutin granules (metachromatic granules): these are mainly

composed of polymerised inorganic phosphate, stored in the cytoplasm as polymetaphosphate which acts as an energy source for the cell metabolism. (b) Lipid granules: these are mainly consist of polymerised β-hydroxybutyric acid and they may act as 'storehouse' for energy and carbon. (c) Polysaccharide granules: these are either glycogen (red-brown) or starch (blue) which can be seen in the cytoplasm of some bacteria when stained with iodine.

Flagella and pili (fimbria)

Flagella. Motile bacteria have thread-like appendages called flagella. A flagellum is a hollow cylindrical structure composed of a protein subunit called flagellin (Macnab and Aizawa, 1984; Doetsch and Sjoblad, 1980). Flagella are the organs of locomotion and if they are mechanically removed, new flagella are formed and the motility is restored within 3-6 minutes. Movement is brought about by the rotation of the flagella. There are 3 patterns of flagella: (a) a single polar flagellum (monotrichous); (b) a group of polar flagella (lophotrichous); (c) flagella distributed over the whole cell (peritrichous). Bacteria respond to changes in the environment by a mechanism called **sensory transduction** (Tayler, 1983). Sensory transduction may occur due to chemotaxis, phototaxis (movement towards light) or aerotaxis (movement towards optimum oxygen concentration) (Boyd and Siman, 1980).

Pili (Fimbria). These are short filaments composed of a protein subunit called pilin and are found in many gram negative bacteria e.g. enterotoxigenic *E. coli*. There are 2 types of pili: (a) sex pili which are responsible for transfer of genes from a donor to a recipient cell during bacterial conjugation; (b) ordinary pili which are responsible for adherence of bacterial cells to host cells. These are, in certain pathogenic bacteria such as enterotoxigenic *E. coli*, known as colonisation antigens (Beachey, 1980).

Sporulation (spore formation)

When the vegetative forms of some bacteria such as *Bacillus* and *Clostridium* meet adverse environmental conditions, especially nutritional depletion such as unavailability of carbon and nitrogen, they form **endospores**. Spore formation involves changes in the enzyme activity and morphology of the cells (Hurst and Gould, 1984). Spores are resting cells which are highly resistant to dehydration, disinfectant, and temperature extremes (Gould and Dring, 1974). Spores, however, are unable to multiply. When favourable conditions return, the spores germinate to form a single vegetative bacterial cell which is capable of reproduction (Keynan, 1973).

Protoplast, Spheroplast and L-Form

Removal of the bacterial cell wall by lysozyme or by antibiotics, such as penicillin, in osmotically suitable media, results in formation of protoplast in Gram-positive bacteria and spheroplast in Gram-negative bacteria. When these cells are cultivated in special culture media with a high osmotic pressure, they form mutant L-form bacteria without cell walls. Some bacteria form the L-form spontaneously. Spontaneously formed or antibiotic-induced L-forms in the host may lead to chronic infection since some L-forms are able to resume normal cell wall synthesis and reproduce if the environment becomes favourable (Clasener, 1972).

Basic structure of bacterial cell walls

Bacteria are classified as Gram-positive and Gram-negative according to their response to the gram staining technique (Cheesbrough, 1984). Bacteria that retain the stain crystal violet are called Gram-positive while bacteria decolorized by washing with acetone or alcohol which then take up the red counterstain are called Gram-negative. The difference in Gram staining of bacteria is, in fact, due to differences in the structure of the cell walls which determine whether the Gram stain is lost or retained. The three different types of bacterial cell walls found in Eubacteria are described below.

(A) Gram-positive bacteria. The cell wall is composed of a thick, cross-linked, peptidoglycan layer and of teichoic acid which consists of polymers of ribitol phosphate and/or glycerol phosphate (Schleifer and Kandler, 1972).

(B) Gram-negative bacteria. The cell wall is composed of a thin, cross-linked, peptidoglycan layer and lipoprotein (Fig. 2.II). The outer layer of the cell wall contains the lipopolysaccharide molecules which are referred to as **endotoxins**. The lipid A part of the lipopolysaccharide is the site of the endotoxin activity i.e. it is the toxic part of the lipopolysaccharide (Osborn, 1969).

(C) Mycoplasmas. They have no cell walls.

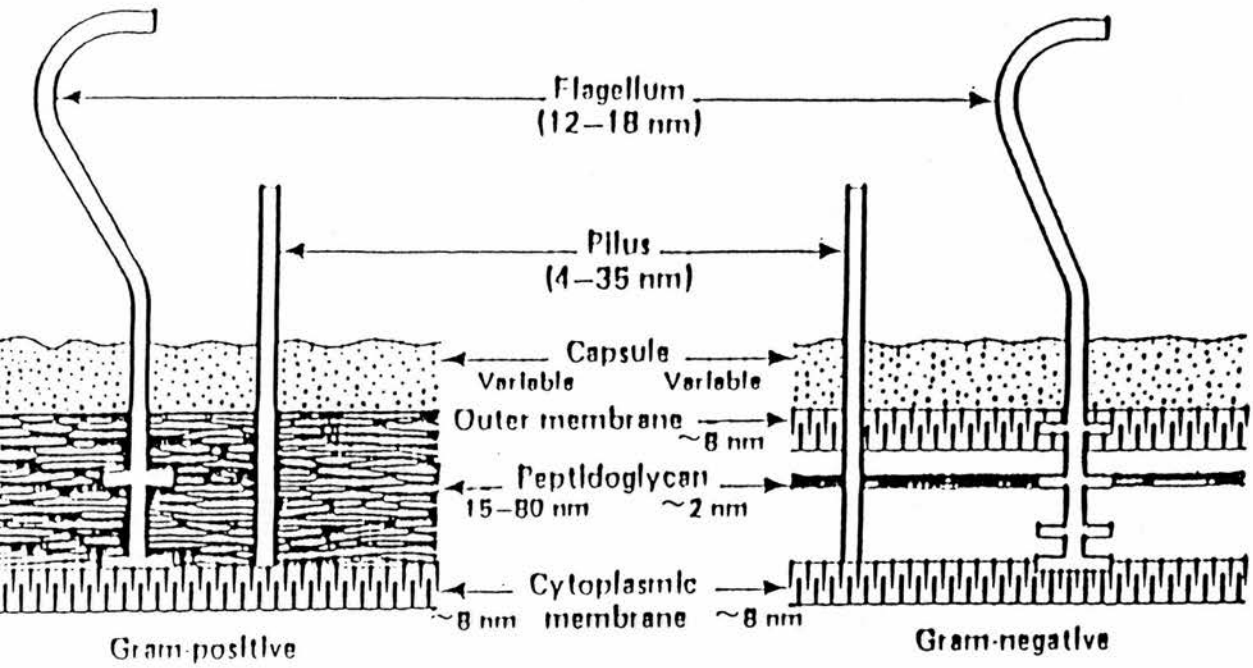


Fig.2.II Comparison between Gram-positive and Gram-negative cell wall structures. (From Jawetz et al, 1987.)

The peptidoglycan layer

Bacterial cell wall is composed of the layers of the cell envelope lying between the cytoplasmic membrane and the capsule. Bacterial cell wall acquires its strength from the peptidoglycan layer (Henning, 1975). The peptidoglycan layer (known also as mucopeptide or murein: all are synonyms) is composed of 3 parts; the glycan backbone, the linear tetrapeptide side chains, and a set of identical cross-linking peptide chains (X). The glycan backbone consists of alternating disaccharide units (N-acetylglucosamine and N-acetylmuramic acid) polymerised by β-1,4 glycoside linkages. The basic structure of the glycan backbone is the same in all bacterial species, but there is marked variations in the polymer length according to numbers of repeating disaccharide units (e.g. *E. coli* has a length of 20-200 units of with an average of 35 units) (Hancock and Poxton, 1988).

The tetrapeptide side chains of the peptidoglycan of different bacterial species have some features in common. These are the L-alanine at position 1, D-glutamic acid at position 2 and D-alanine at position 4. Position 3 is a variable diamino acid and is species specific. In most Gram-negative bacteria, diaminopimelic acid (DAP) occupies position 3, while in Gram-positive bacteria L-lysine, diaminopimelic acid or any L-amino acid may occupy position 3 (Jawetz et al, 1987).

The cross-linkages vary from species to species. In most Gram-negative bacteria the cross-linkages form direct bridges between the amino group of diaminopimelic

acid of one tetrapeptide side chain and the carboxyl group of the terminal D-alanine. In many Gram-positive bacteria e.g. *Staph. aureus,* pentaglycine peptide chains (5gly) cross-linkages connect the amino group of the L-lysine of one side chain to the carboxyl group of the terminal D-alanine of the next chain .

Special features of Gram-negative cell walls

As cited above, the cell wall of Gram-negative bacteria contains lipoprotein, outer membrane and lipopolysaccharide, in addition to the peptidoglycan layer.

The Lipoprotein

Lipoprotein is the most abundant protein of Gram-negative cells. The protein component contains 57 amino acids, representing repeats of a 15-amino-acid sequence (Inouye, 1979). Lipoprotein is covalently linked to the diaminopimelic acid residues of the peptidoglycan through its C-terminal lysine residue and links the peptidoglycan layer to the outer membrane. Lipoprotein stabilises the outer membrane and anchors it to the peptidoglycan layer.

The Outer membrane

This is a bilayer structure of which a phospholipid layer forms the inner layer while the outer layer is formed by the lipopolysaccharide molecules (Inouye, 1980). Proteins of the outer membrane are individually named after the genes that encode them. In *E. coli* and *Salmonella typhimurium* the **porin** proteins OmpC, D and F penetrate both faces of the outer membrane and form relatively non-specific pores that allow diffusion of small hydrophilic solutes through the membrane. In addition to its function as a barrier preventing the leakage of the periplasmic proteins, the outer membrane protects the bacterial cell (in enteric bacteria) from bile salts and hydrolytic enzymes. The protein pores render the outer membrane permeable to low-molecular weight solutes while higher molecular weight molecules, such as antibiotics, pass relatively slowly. This may account for the high antibiotic resistance of Gram-negative bacteria in which the outer membrane is less permeable to large antibiotic molecules.

The Lipopolysaccharide (LPS)

The lipopolysaccharide of Gram-negative bacteria is usually composed of 3 main parts: lipid A, core oligosaccharide and a series of repeating units (O antigen polysaccharide or O-specific chain) (Fig. 2.III).

Lipid A is composed of phosphorylated β-1,6-linked D-glucosamine disaccharide backbone to which are attached a number of long-chain fatty acids (Takayama et al, 1983; Stewart-Tull and Davis, 1985). Lipid A has a unique fatty acid called β-hydroxymyristic acid while other fatty acids vary according to the bacterial species. Lipid A is often highly toxic and it is the biologically active part of the lipopolysaccharide. It is usually referred to as **endotoxin** because it is firmly attached to the cell surface and was thought to be released only when the cell is lysed. However, it was found that a considerable amount of endotoxin may be released by intact and actively growing bacteria (Galanos et al, 1977).

The core oligosaccharide is composed of a branched oligosaccharide containing a unique sugar called 2-keto-3-deoxy-D-manno-octonate (KDO) in addition to heptose, phosphoethanolamine and several hexoses. The structure of the core oligosaccharide is present in all Gram-negative bacteria and is group-specific (Luderitz et al, 1966).

The O-specific chain (O antigen) is a polymer of repeating units of oligosaccharide (usually linear trisaccharides or branched tetra- or pentasaccharides) molecules and represents a major surface antigen of bacterial cells. The O antigen polysaccharide is serotype-specific and this character was used in the Kauffmann-White classification of *Salmonella* spp. (Luderitz et al, 1966). LPS is highly immunogenic inducing specific antibodies against the terminal repeating sugar units

of the polysaccharide part (O-specific polysaccharide) (Stewart-Tull and Davis, 1985).

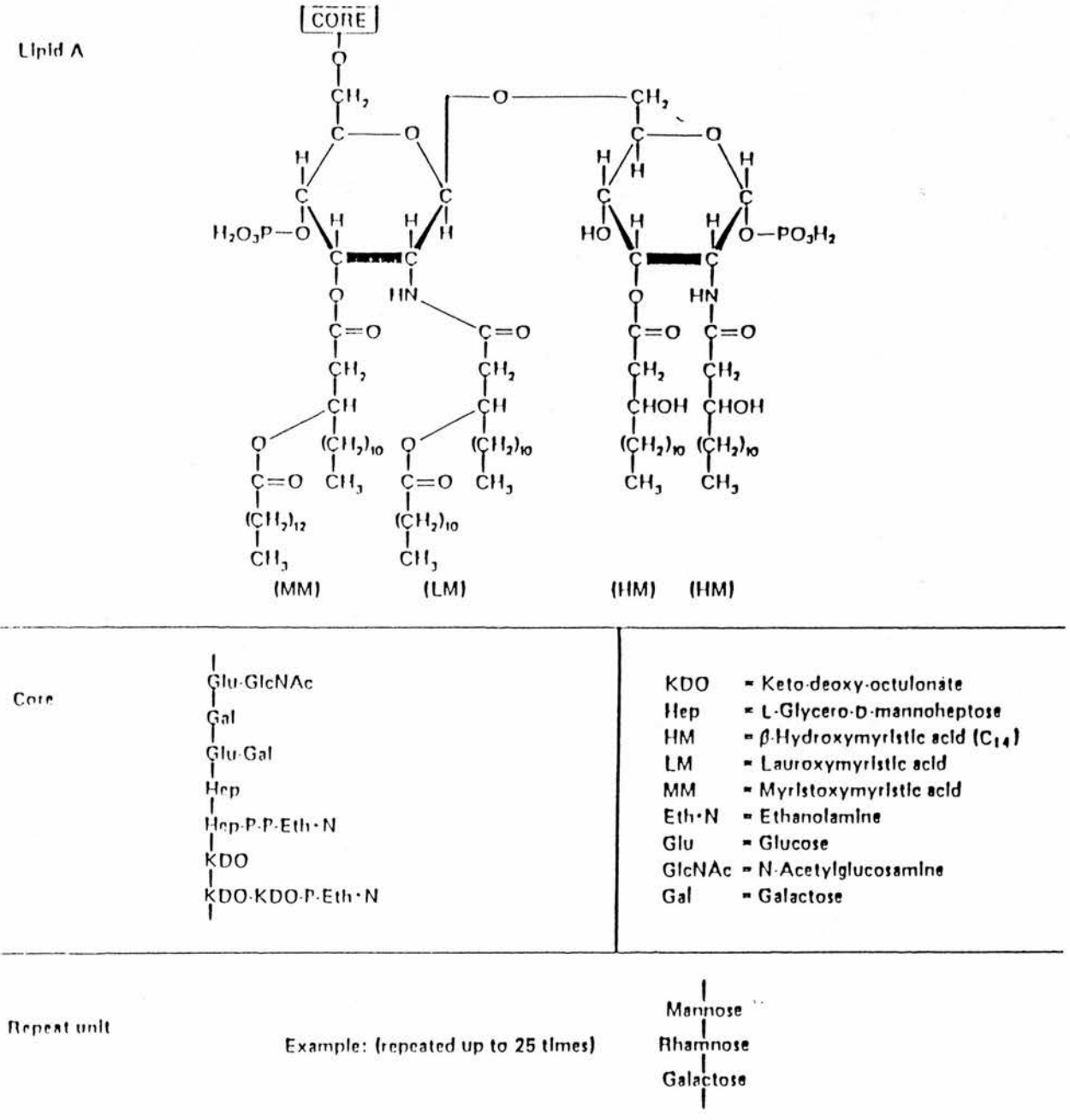


Fig. 2.III The 3 main components of the LPS of Gram-negative bacteria. (From Jawetz et al, 1987.)

The pathophysiological effects of LPS on the mammalian body

Fever. The thermoregulatory centre of the hypothalamus maintains the body temperature within the normal limits due to balance between heat production and heat loss. Endotoxins produced by Gram-negative bacteria such as *Salmonella*, *Shigella*, *E. coli* etc. act on monocytes and macrophages resulting in the release of interleukin-1 (endogenous pyrogen) which acts on the thermoregulatory centres of the hypothalamus causing fever (Dinarella and Wolff, 1982). Repeated injection of endotoxin, however, resulted in decreasing fever response may be due to tolerance.

Changes in leukocyte counts. Endotoxin results in an initial **leucopenia** due to the sequestration of neutrophils in the capillary beds. This is followed by a leucocytosis due to the release of granulocytes from bone marrow (Athens et al, 1961). Endotoxins increase the phagocytic and metabolic activities (glycolysis) of many cells resulting in hypoglycaemia (Morrison and Ulevitch, 1978).

Vascular and circulatory changes. Infection with Gram-negative bacteria results in an initial vasoconstriction of the arterioles and venules followed by peripheral vascular dilatation. Endotoxins can activate the release of vasoactive substances such as serotonin and kinins, resulting in the above vascular changes (Jawetz et al, 1987). Endotoxin can also activate both the intrinsic and extrinsic pathways of coagulation (Morrison and Ulevitch, 1978). They activate Hageman Factor (Factor XII) initiating the first step of the intrinsic pathway of coagulation. Endotoxins also

act on the leukocytes (mostly the monocytes), for which process lipid A is required, triggering the first step in the extrinsic pathway of coagulation (Rivers et al, 1975). Intrinsic and extrinsic pathways lead to conversion of fibrinogen into fibrin causing disseminated intravascular coagulation which is a frequent complication of Gram-negative bacterial infection.

Shwartzman reaction. Animal experiments showed that when a rabbit was injected intradermally with endotoxin followed 24 hours later by an intravenous injection of another dose of endotoxin, this resulted in a necrosis at the site of intradermal injection. This phenomenon is called the **local Shwartzman reaction** (Shwartzman, 1937). When endotoxin was given intravenously on 2 successive days, this resulted in the development of disseminated intravascular coagulation. This is called the **generalised Shwartzman reaction.** It has been suggested that the first dose of endotoxin blocks the reticuloendothelial system so that it is not able to clear the second dose of endotoxin (Stewart-Tull and Davis, 1985).

Mechanism of action of LPS as an endotoxin

There is now much evidence that the endotoxic effect of LPS on target organs is mediated by a variety of cytokines and other soluble factors. Macrophages have been found to play an important role in the mechanism by which LPS produces its endotoxic effect when stimulated by LPS (Manthey and Vogel, 1992). In

experimental animals, purified LPS induced the production of tumour necrosis factor (TNF), interleukin 6 (IL-6), interferon (IFN) and colony stimulating factor (CSF) with the TNF peaked very early (about one hour after injection of LPS) while CSF peaked few hours later (about 6 hours after injection of LPS) (Henricson et al, 1990). In addition to TNF, IL-1 is an LPS-induced cytokine that has been found to be produced by macrophages and appears early in the cascade of events that results in manifestations of endotoxicity (Fong et al, 1989). The latter study suggested that TNF may be an important factor that regulate the production of LPS-induced IL-1.

Once macrophages are stimulated and produced TNF and IL-1, a co-ordination between these two mediators appears to mediate the steps that follow in the cascade of cytokines production. For instance, *in vivo* injection of animals with IL-1 stimulated the production of IL-6 (Neta et al, 1988) while pre-treatment of animals with anti-TNF antibodies markedly inhibited the production of IL-6 (Fong et al, 1989).

The production of CSF as a response to stimulation by LPS seems to be markedly induced by TNF and IL-1. Vogel and colleagues (Vogel et al, 1987) reported that recombinant TNF or IL-1α stimulated the production of CSF more rapidly than did a dose of LPS of 25 μg. CSF may be produced by stromal cells rather than macrophages. This is because nude mice, which lack T cells, have been found to

respond normally and produces CSF as a response to LPS injection (Manthey and Vogel, 1992).

The recent development in the techniques used for detection of cytokines and other mediators has led to detection of more cytokines and other mediators that may be involved in the cascade of events produced by LPS endotoxicity. For example, in addition to the previously mentioned cytokines, IL-8 and IFN-γ have recently been found to be produced, in animals, as a response to LPS injection (Van Zee et al, 1991; Heinzel, 1990). Furthermore, LPS has been found to stimulate inflammatory cells to express a variety of lipid autocoids such as leukotrienes, prostaglandins and platelet activating factor (PAF) (Manthey and Vogel, 1992). PAF is thought to enhance the production and expression of cytokines while prostaglandin E_2 (PGE_2) is thought to have depressant effect.

Mechanism of action of LPS in septic shock and tissue injury. The lethal effect of LPS on tissues has been found to be mainly due to neutrophils infiltration and ischaemia. Manthey and Vogel (1992) have suggested that LPS produces cardiovascular shock by stimulating the expression of the intercellular adhesion molecule 1 (ICAM1) on the surface of the vascular endothelial cells which binds CR3 and LFA-1 antigens expressed on the surface of neutrophils. Cytokines stimulated by LPS induce endothelial and stromal cells to release chemotactic factors that attract neutrophils to pass through the vascular endothelium and infiltrate tissues. Locally produced prostaglandins and PAF induce vasodilatation

resulting in hyperaemia and capillary leakage. Endothelial and macrophages are stimulated to express procoagulant activity resulting in the formation of intravascular fibrin clots, blocking the circulation and leading to local ischaemia.

Genetic resistance to LPS

Sultzer (1968) found that the mouse strain C3H/HeJ is resistant to some of the endotoxic effects of LPS. Later, Watson et al (1974) found that a single locus on chromosome 4 regulates a number of endotoxic reactions induced by lipid A. This locus was called **LPS responsiveness locus**. The functions of the LPS responsiveness locus were suggested by Watson et al (1980) as follows: the LPS locus may control genes coding for the cellular receptor for lipid A or may control genes coding for expression of cellular components required to convert the binding process into biochemical signals. The macrophages and lymphocytes of the mouse strain C3H/HeJ did not respond to LPS and this was found to be due to the absence of specific receptors on these cells (Morrison and Ryan, 1979; Forui and Coutinho, 1978).

Antigenic structure and antigenic properties of enteric Gram-negative bacilli (Enterobacteriaceae)

Enterobacteriaceae have a complex antigenic structure. In addition to the somatic (O), flagellar (H) and the capsular (K) (found on *Salmonella typhi*) antigens mentioned before, fimbrial antigen (Type-1 fimbria) is formed by most strains of salmonellae and leads to confusing cross-reaction in the agglutination test. Likewise, M antigen (the slime or mucous antigen) prevents agglutination by anti-O antigen antibodies (Sleigh and Duguid, 1989).

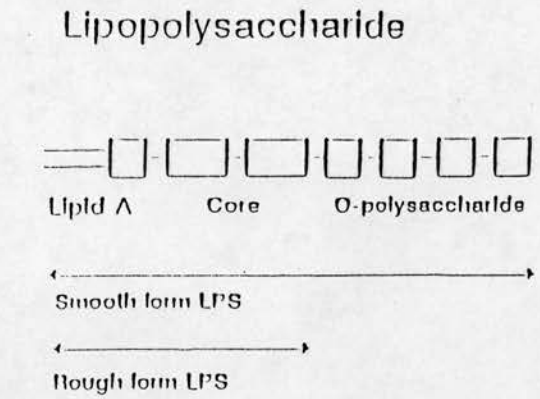
O antigen (somatic antigen). This antigen represents the side-chain of the repeating units of the lipopolysaccharide. It is heat-stable and resistant to alcohol (withstanding treatment with 96% ethanol for 4 hours at 37 °C). There are more than 150 different O somatic antigens possessed by the different genera of the Enterobacteriaceae and a single organism may possess more than one O antigen, thus one (or more) O antigen could be common to various strains of *Salmonella*, *Shigella* and *E. coli* leading to cross-reaction among these strains in the agglutination reaction test. However, O antigen is used in subgrouping of the different genera of Enterobacteriaceae. Although IgM antibodies are the predominant antibodies against O antigen (10), yet IgG and not IgM antibodies were associated with the decrease in morbidity and mortality due to Gram-negative bacteraemia (McCabe et al, 1972) and although antibodies against antigen protected animals in experimental infections with gram negative bacteria (Kaijser and Olling,

1973), yet until recently there was no practical evidence to confirm these findings in humans.

R antigen. All cultures of Enterobacteriaceae contain variant and stable mutants with respect to colonial morphology, antigenic characteristics and virus resistance. Regarding their colonial morphology, these mutants could be in the smooth (S) or in the rough (R) form. In S→R mutation, the O-specific side-chain (O antigen) is lost and the remaining core linked to the lipid A forms a new rough (R) antigen. There are many types of R mutants (Fig. 2.IV): (1) Ra mutants: they lack the O antigen but have the complete core attached to lipid A; (2) Rb to Re mutants: these mutants have an incomplete core and they bear core fragments of varying length according to the step at which the biosynthesis was blocked (Galanos et al, 1969).

It has been found that antibodies against O antigen are specific for individual LPS (Galanos et al, 1977). Thus antibodies against the smooth (S) bacteria (parent bacteria with complete LPS) are not effective in protecting from infection by unrelated Gram-negative bacteria. On the other hand, anti-R and anti-lipid A antibodies were found to be directed against a structure common to different LPS. This common immunogenic character of R mutants was employed in developing a cross-protective immunisation against a number of Gram-negative bacteria in animals (Brande et al, 1977) and also in reducing the morbidity and mortality due to Gram-negative bacteraemia in human volunteers compared to controls (Ziegler et al, 1982).

Gram-negative bacteria are present in large numbers as a commensal flora in man e.g. in the oropharynx and in the urogenital tract, and colonise the gastrointestinal tract from birth onwards. However, it is not yet known whether this continuous presence of Gram-negative bacteria affects the response to LPS or not. In animals, *in vitro* experiments showed that germ-free animals had stronger immunological responses to LPS (Kiyono et al, 1980), and another study (Schaedler and Dubs, 1961) showed that they had higher resistance against the lethal effects of LPS, than conventional animals. These observations suggest that the commensal flora produces immunologic tolerance to endotoxin and hence, decreases the resistance to pathogenic bacteria. Although LPS has been shown to be an important factor in the virulence of the Gram-negative bacteria, yet possession of LPS in itself does not confer pathogenicity on the bacteria. In fact, LPS is a component of the cell envelopes of pathogenic, non-pathogenic and saprophytic (micro-organisms living upon dead or decaying organic matter) Gram-negative bacteria. Furthermore, LPS is not the only toxic factor in the pathogenicity of many Gram-negative bacteria, especially those which produce exotoxin or those which have invasive capability.



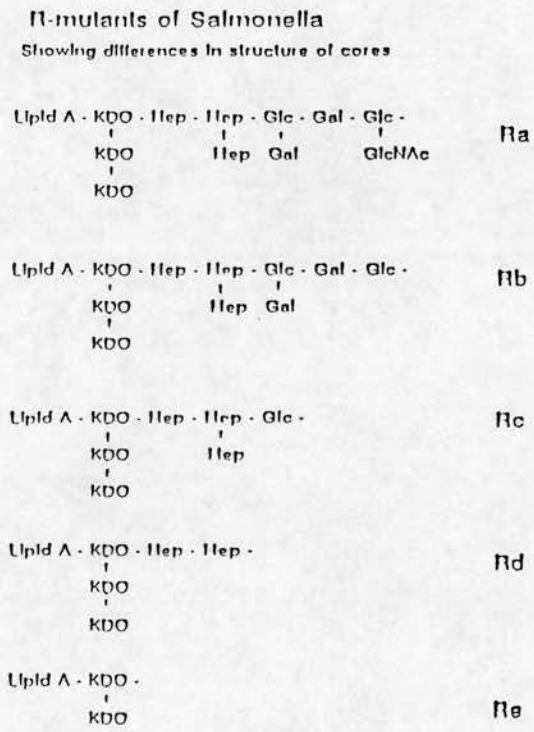


Fig. 2.IV: (a) Diagrammatic representation of LPS of *Salmonella*. When the O-polysaccharide is lost through mutation the LPS is termed "rough", compared to the "smooth" wild-type parent. (b) Rough mutants of *Salmonella*. The Ra mutant produces LPS with a complete core but no O-polysaccharide. Other R-mutants (Rb-Re) produce only partial cores. The Re-mutant is the minimum LPS structure which permits viability of the organism. KDO=keto-deoxy-octanic acid, Hep=heptose, Glc=glucose, Gal=galactose and GlcNAc=N-acetyl glucosamine.

H antigen (flagellar antigen). This antigen is heat and alcohol-labile and heating for 30 minutes at 100 °C will remove all the flagella from the bacteria. The unflagellated bacteria cannot be agglutinated by anti-H antibodies. Anti-H antibodies are mainly IgG (Jawetz et al, 1987). H antigen on the bacterial surface may interfere with agglutination by anti-O antibodies. The amino acid sequences in the flagellar protein (flagellin) are responsible for the production of H antigen (Jawetz et al, 1987). Within a single serotype of a genus of Enterobacteriaceae, flagellar antigens may be present in either or both of 2 forms, called phase 1 and phase 2. In phase 1, known also as the 'specific phase', the bacterium has one or more antigen from a set of 70 H antigens designated by small letter of the alphabet, a to z then z1, z2, z3 etc. In phase 2, known as the 'group' or 'non-specific phase', the bacterium has one or more antigens from another set of H antigens designated by Arabic numerals. The strains vary spontaneously and reversibly between these 2 phases. Thus the antigenic classification of Enterobacteriaceae often indicates the presence of specific antigens e.g. in *Salmonella paratyphi* B it is O1,4, 5,12:Hb1,2.

K antigen (capsular antigen). Some strains of Enterobacteriaceae produce a thick capsule surrounding the bacterial cell formed of polysaccharide or proteins and known as K antigen. In *Salmonella typhi* this antigen is called Vi antigen. K antigen is a heat-labile antigen and can be removed from the bacteria by heating a suspension for 1 hour at 100 °C. This antigen may be associated with the virulence of the organism e.g. in *E. coli* attachment of the bacteria to the epithelial cells

before the invasion of the gastrointestinal or the urinary tract is facilitated by K antigen, and in *Salmonella typhi* it was found that Vi antigen is associated with invasiveness of the organism. K antigen may interfere with agglutination by anti-O antibodies (Morrison and Ryan, 1979).

Section 4

VACCINATION AGAINST TYPHOID FEVER

Introduction

Control of enteric infections can be achieved by improvement of primary health care such as improvement of water supply, sanitation and health education. However, because of the limitation of resources and other competing priorities in many parts of the world, development of vaccines against enteric pathogens may help in the control of the wide range of infectious diarrhoea and other enteric infections.

Typhoid fever is not a diarrhoeal disease *per se* but vaccines against this enteric infection are important for several reasons: (a) it is one of the most serious health problems in many developing countries with an incidence rate of about 33 million cases per year world-wide (Simanjuntak et al, 1991); (b) in all endemic areas, the highest incidence is concentrated among school-age children (5-19 years old) (Levine et al, 1989); (c) appearance of multiple drug-resistant strains of *Salmonella typhi* to the main clinically important antibiotics world-wide (Rowe et al, 1990); (d) the new vaccines against typhoid fever may serve as a live carriers for vaccines against other infectious agents that cause diarrhoea e.g. *Shigella, V. Cholerae* O1 and rotavirus (Levine and Noriega, 1993; Forrest et al, 1989).

Parenteral typhoid vaccines

In 1896, Pfeiffer and Wright introduced the first parenteral vaccine for human use against typhoid fever. They independently reported that a vaccine against typhoid fever could be prepared by inactivating a culture of typhoid bacilli with heat and preserving with phenol (Pfeiffer and Kolle, 1896; Wright, 1896; Groschel and Hornick, 1981). However, a series of large-scale field trials in several countries sponsored by the World Health Organisation (WHO) to assess the efficacy of various types of parenteral killed whole-cell vaccines was only carried out in the 1950s and 1960s (Yugoslav Typhoid Commission, 1957; Yugoslav Typhoid Commission, 1964; Polish Typhoid Commission, 1966; Hejfect et al, 1966; Aschcroft et al, 1967). Three types of parenteral killed whole-cell vaccines were assessed: (a) alcohol-inactivated; (b) heat-inactivated and phenol preserved; (c) acetone-inactivated vaccines. These vaccines conferred 60%, 70-75% and 70-85% protection for 1-2 years, 3 years and 3-4 years respectively (World Health Organisation, 1979). Administration of the parenteral killed whole-cell vaccines against typhoid and para typhoid fever A and B (known as TAB vaccine) has been associated with systemic (mainly fever) and local (pain) effects in 25-50% of the vaccinees (Levine et al, 1989). Because TAB vaccines only provide partial protection and also because of the high rate of adverse reactions associated with their administration, they rarely have been used in any country, with the exception of Thailand, for systematic typhoid fever control programs (Levine et al, 1989). Therefore, the search for

a more acceptable and satisfactory vaccine continued and recently new vaccines against typhoid fever, namely the oral typhoid vaccine Ty21a and the parenteral vaccine, Typhim Vi, have been developed.

Oral typhoid vaccines

It is now almost axiomatic that vaccination against enteric infections such as typhoid fever has to be at the portal of entry of the infectious agents. The aim of oral administration of vaccines is to stimulate local intestinal immune response, especially of the secretory immunoglobulin A (SIgA) class. Induction of strong local immune responses is necessary for the efficacy of vaccines against enteric infections (World Health Organisation, 1979; World Health Organisation, 1982). SIgA may provide antibacterial protection by two mechanisms: (a) direct action on bacteria, which may result in immobilisation, agglutination, or prevention of adherence to the mucosa; and (b) combination with bacterial products such as toxins or enzymes which cause inactivation and help in their destruction by proteolytic enzymes (World Health Organisation, 1979). In addition to the fact that the route of antigen administration is important in the development of an appropriate strong immune response, oral vaccines are generally easier to produce and control for quality than parenteral vaccines. Furthermore, oral vaccines are less reactogenic i.e. less side effects, easier to administer and do not require medically trained personnel or sterile

equipment. Based on the concept of the common mucosal immune system, whereby a local presentation of an antigen at one mucosal surface can stimulate an immune response at distant mucosal surfaces (Mestecky et al, 1978; Weisz-Carrington et al, 1979; Mestecky, 1987; Weisz-Carrington et al, 1987) oral vaccination has become an area of much interest because of the possibility of development of oral vaccines not only against enteric infections but also against other infections in other areas of the human body such as the respiratory system (Ogra and Karzon, 1969; Forrest et al, 1990).

The oral typhoid vaccine Ty21a

The live attenuated oral typhoid vaccine Ty21a was developed by chemical mutagenesis of the pathogenic strain of *Salmonella typhi* in the early 1970s and proved to be safe (with mild, if any, adverse reactions) and genetically stable (Germanier and Furer, 1975; Gilman et al, 1977). Large-scale field trials carried out in Egypt, Chile and Indonesia measuring the protective efficacy of the vaccine Ty21a have shown significant although variable levels of protection against typhoid fever (Wahdan et al, 1980; Wahdan et al, 1982; Levine et al, 1987; Simanjuntak et al, 1991). The protection rates were 96%, 67%, and 42% in Egypt, Chile and Indonesia respectively. The variation in the rates of protection were attributed to the differences in the formulations, vaccination schedules and the incidence rates of infection in these countries.

The incidence rates of infection among children aged 5-19 years were $50/10^5$, $150/10^5$ and more than $1000/10^5$ in Egypt, Chile and Indonesia respectively (Simanjuntak et al, 1991).

Genetic characteristics of the vaccine Ty21a

In the early 1970s, Germanier and Furer reported the isolation and characterisation of a *Salmonella typhi* strain as a possible candidate for a live oral vaccine against typhoid fever (Germanier and Furer, 1975). In animals, an avirulent mutant of *Salmonella typhimurim* called gal E mutant was found to be suitable to be used as a live vaccine and provided mice with protection against sublethal infection with a virulent strain of *S. typhimurium* (Germanier and Furer, 1975). Gal E mutant is a rough-type strain that is unable to synthesise a complete lipopolysaccharide in the absence of galactose in the medium (Germanier and Furer, 1971). This defect is caused by the absence of an enzyme called Uridine Diphosphate (UDP)-galactose-4-epimerase due to the chemical mutagenesis (Nikaido, 1961; Germanier and Furer, 1975). The protectivity of the vaccine is due to the fact that when galactose is supplied exogenously (as occurs in vivo), the mutant regains its ability to synthesise lipopolysaccharide, the immunologically important cell wall of smooth-type strains, and to form the protective immunity (Germanier and Furer, 1975). The avirulence of gal E mutant strains is mainly due to strong bacterial lysis

following the uptake of galactose which when metabolised will result in accumulation of galactose-1-phosphate and UDP-galactose (Fukasawa and Nikaido, 1961). The avirulence of gal E mutant strains was also attributed to the incomplete cell wall lipopolysaccharide (Germanier and Furer, 1975).

Based on the results obtained with *S. typhimurium* in mice, gal E mutant *S. typhi* strains were isolated after chemical mutagenesis of the parent strain Ty2 of *S. typhi* (Germanier and Furer, 1975). Of several gal E mutants of *S. typhi*, Ty21a was found to be genetically stable; reversion to the wild type (smooth type) was not observed *in vitro* or *in vivo*. Furthermore, the vaccine strain of *S. typhi* lacks the capsular antigen Vi which is known as the virulence factor in the pathogenic strains of *S. typhi* (Silva et al, 1978). When given as an oral typhoid vaccine (five to eight doses containing 3-10 x 10^{10} viable organisms) to healthy volunteers from a non-endemic area, Ty21a provided 87% clinical protection against experimental challenge with 10^{5} virulent *S. typhi* (Gilman and Hornick, 1977).

Determinants of mucosal immune response to vaccination

There are many factors that may influence the development of the mucosal immune response to vaccines. As mentioned above, the route of administration of a vaccine is an important factor that influences the outcome of vaccination.

Studies have shown that oral rather than parenteral administration of the vaccines, produces a strong mucosal immune responses (Ogra et al, 1968; Heremans, 1974; Ogra et al, 1976; Lange and Holmgren, 1978; Svennerholm et al, 1980; Mestecky et al, 1986). Furthermore, studies showed that parenteral immunisation may actually suppress mucosal immune response and the SIgA production (Pierce, 1980; Pierce, 1984). Oral vaccination followed by oral boosting resulted in a good mucosal immune response and oral vaccination followed by parenteral boosting gave rise to an intermediate results while parenteral vaccination followed by parenteral boosting resulted in mucosal tolerance rather than immunity (Svennerholm et al, 1984a; Holmgren and Lycke, 1986).

Another important factor that may influence the outcome of immunisation is the previous exposure to the same antigen or a cross-reacting antigen at the mucosal site. It seems that previous exposure to the same antigen or cross-reacting antigen by vaccination or natural infection primes the mucosal immune system (Svennerholm et al, 1977; Svennerholm et al, 1980; Svennerholm et al, 1984a). For instance, Swedish women failed to produce specific mucosal antibodies following parenteral cholera immunisation while such vaccine stimulated a specific SIgA antibody response in breast milk of Pakistani women (Svennerholm et al, 1980). Another example of the importance of previous exposure to the challenging or cross-reacting antigen is the lack of the immune response to an orally administered vaccine in young children. Studies in young

children (infants and toddlers 6-24 months of age) showed that administration of oral *S. typhi* vaccine Ty21a did not produce any immune response while administration of the same vaccine to school-age children produced significant increase in the immune response (Murphy et al, 1991).

The nature of the challenging antigen is another important determinant of the immune response to a vaccine. Antigens which are not degraded in the intestine can bind to the apical surface of M cells of PPs and, with adjuvant activity, such as cholera toxins, usually stimulate a good intestinal immune response (Elson and Ealding, 1984; Lycke and Holmgren, 1986). Most food antigens, on the other hand, are poor mucosal immunogens because they are rapidly degraded in the intestine into non-immunogenic substances which do not bind to M cells of PPs and may have tolerogenic rather than adjuvant characters (Brandtzaeg et al, 1990; Lycke and Svennerholm, 1990). Live vaccines can produce stronger mucosal immune response following a single oral vaccination than can killed vaccines (Ogra et al, 1968; Levine, 1989). Following a single antigenic challenge, primed cells may migrate into the lamina propria and only limited cell proliferation and SIgA synthesis occurs (Brandtzaeg et al, 1990; Lycke and Svennerholm, 1990). Therefore, it seems that live vaccines provide sustained antigenic stimulation of the immune response over many years.

The frequency, doses, formulation and timing of immunisation may also have a significant influence on the immune response. In field trials in Chile, three

doses of the oral typhoid vaccine Ty21a in the form of enteric-coated capsules provided 67% protection for at least three years and four doses of the same vaccine in the same formulation provided significantly greater protection than did three doses (Levine, 1989). In contrast, in the above study one or two doses of the vaccine Ty21a as enteric-coated capsules conferred lower level of protection for only two years. Therefore, repeated and continuous antigenic challenge of the gut mucosa by multiple doses of live vaccine seems to stimulate a stronger immune response than a single exposure of an inactivated antigen.

The dose of the vaccine required to stimulate an immune response may be a key factor influencing the outcome of vaccination. While the oral typhoid vaccine Ty21a at doses containing 10^9 viable *S. typhi* have been shown to confer a significant protection against typhoid fever (Wahdan et al, 1980; Wahdan et al, 1982), other studies showed that increasing the dose to contain up to 10^{10} or 10^{11} viable organisms will produce stronger immune response both in intestinal mucosa and in serum (Forrest et al, 1991).

Different formulations of the same vaccine seem to produce different protective efficacy when used at the same doses. Field trials showed that the vaccine Ty21a in enteric-coated capsules gave a higher protective efficacy than did in gelatine capsules/sodium bicarbonate formulation (Levine et al, 1987). However, other field trials showed that the vaccine Ty21a in liquid formulation

was preferable to enteric-coated capsules and produced higher protection than did the enteric coated capsules (Levine et al, 1990, Simanjuntak et al, 1991).

Optimal timing of the repeated antigen challenge is also an important factor that may influence the outcome of the vaccination. Studies showed that repeated doses of the vaccine at intervals consistent with the build-up of the mucosal immunological memory may optimise the immune response (Lycke and Svennerholm, 1990). Based on the results obtained by Forrest et al (1991) regarding the optimal timing for administering the oral typhoid vaccine Ty21a and studying the intestinal immune responses to the vaccine, I designed the study of intestinal immune response to the vaccine Ty21a in British healthy volunteers (see later).

Section 5

SMOKING AND THE INTESTINAL IMMUNE SYSTEM

Introduction

The association of tobacco smoking with the prevalence of a variety of diseases in the respiratory tract and in distant organs of the human body is well known (World Health Organisation, 1975; US. Department of Health and Education Welfare, 1979; US. Department of Health and Human Services, 1983). The effects of tobacco smoking on the gastrointestinal tract have been the subject of several studies (Packard, 1960; Bennett, 1972; Kikendall et al, 1984). However, most of these studies only addressed the effects of tobacco smoking on the upper part of the gastrointestinal tract but not the intestine.

The effects of tobacco smoking on immunity in animals and humans have been extensively studied by Holt and Keast (1977) and later comprehensively reviewed by Holt (1987). However, these studies only investigated the effects of smoking on the systemic immune system. Other studies investigated the effects of smoking on the concentration of immunoglobulins in the salivary specimens of a group of healthy control and in patients with head and neck tumours (Barton et al, 1990). However, it is now axiomatic that the systemic immune system is separate from the mucosal immune system and the findings

in saliva do not necessarily represent the immune response status at the intestinal mucosal level (O'Mahony, et al, 1991a).

The appearance of reports suggesting the presence of a relationship between cigarette smoking and inflammatory bowel disease (Harries et al, 1982; Benoni and Nilsson, 1984; Motley et al, 1988; Jick and Walker, 1983; Tobin et al, 1987) has increased the interest on the effects of smoking on the intestinal mucosal immune system. Some workers resorted to *in vitro* study of immunoglobulin production in the colonic mucosa of two groups of controls (smokers and non-smokers) and patients with inflammatory bowel disease (Cope et al, 1989).

Apart from the study carried out by Srivastava and colleagues (1991), there have been no *in vivo* studies investigating the relationship between smoking and the intestinal mucosal immune system. However, the smoker group in the above study were not heavy smokers and used to smoke only an average of 15 cigarette per day for one year.

Tobacco smoking has been shown to influence a variety of factors that may directly or indirectly affect the integrity of the immune system and subsequently its capability to protect the human body against a wide spectrum of infectious agents. Furthermore, the presence of an integrated immune system

has also been found to be important for the reaction against and removal of new antigenic cells that may develop to malignant cells.

Green (1954) suggested the presence of a specific immune mechanism that may be involved in the control of cancer. This mechanism was called "immunosurveillance mechanism". Later on, Keast (1970) reviewed studies that provided evidence for the possibility of presence of an immunosurveillance mechanism and supported the suggestion by Green (1954) regarding the presence of such mechanism in the normal immune system.

Therefore, factors that may influence the integrity of the immune system such as immunosuppressive drugs, infections, congenital and acquired immune deficiency diseases and cigarette smoking may contribute to the immunosenescence of the immune system.

Studies in animals

Effect of cigarette smoke on humoral immunity

Cigarette smoke has been shown to modify the humoral immune response of the local as well as the systemic immune system of animals. In mice, exposure to heavy cigarette smoke (30 cigarettes/day for 26 weeks) resulted in

suppression of both primary and secondary immune responses against intratracheally injected sheep red blood cells (SRBC) (Thomas et al, 1973). In this study, suppression of the immune responses was not limited to the local immune response in the bronchopulmonary system but extended into distant organs such as the regional lymph nodes and spleen. The degree of immunosuppression was found to be related to the distance of the organ involved from the site of entry of cigarette smoke (Thomas et al, 1973; Holt and Keast, 1977).

Cigarette smoke was shown to produce biphasic changes in the primary immune responses of the regional lymph nodes and spleen of mice with an initial enhancement followed by depression of the immune responses against SRBC. Thomas et al (1974) examined the effect of cigarette smoke on mice for varying periods up to 42 weeks. After 9 days exposure to cigarette smoke (mixture of smoke/air (1:7) at a puff volume of 35 ml) the number of direct and indirect plaque-forming cells (pfc) detectable in the lungs of mice as a response to intratracheally injected SRBC fell to 30% of that in the control animals. After 182 days exposure, the number of pfc in the lungs of mice fell to less than 5% of the normal control. The primary immune response in the lungs was virtually abrogated and the secondary immune response was delayed. Furthermore, the immune response in the lungs was found to be different from the immune responses in distal organs. For instance, the immune response in the lungs exhibited an immediate depression after commencement of exposure

to cigarette smoke and enhancement was never observed. In contrast, the immune responses in the regional lymph nodes and spleen after exposure to cigarette smoke showed biphasic changes with an initial enhancement followed by depression with long-term exposure. In another study (Thomas et al, 1975), similar biphasic changes in the primary immune responses were observed. In that study, however, the secondary immune responses were not significantly changed.

The effect of high tar (HT) and low tar (LT) cigarette on the primary and secondary immune responses of animals were studied by Holt et al (Holt et al, 1976). In mice, exposure to LT cigarette smoke for a period of 26 weeks resulted in enhanced primary followed by normal secondary immune responses against intraperitoneally injected SRBC whereas exposure of mice to HT cigarette smoke for the same period exhibited marked depression of primary and delay in secondary immune responses.

Various contents of tobacco smoke were studied in an attempt to elucidate the effect of exposure to cigarette smoke. In mice, daily intraperitoneal injection of 0.1 ml of water soluble fraction (WSF) of cigarette smoke for 8 weeks resulted in significant decrease in the number of serum pfc and serum concentration of IgG against SRBC compared to the control animals (Yamakido et al, 1984). In another study (Onari et al, 1980), daily subcutaneous injection of 0.1 ml of WSF of cigarette smoke for 6 weeks before being antigenically challenged with

SRBC resulted in a significant inhibition in the increase of serum IgG levels and decrease in the number of IgM-forming cells in the spleen of the injected mice.

Adding concentrations of either nicotine or WSF of cigarette smoke ranging from 1 to 100 μg per ml to a rabbit lymphoid cell culture resulted in a significant suppression of IgG and IgM pfc responses against SRBC (Roszman and Rogers, 1973). In this study, suppression of immune responses was found to be dependent on the concentration of nicotine or WSF and complete suppression of IgG and IgM antibody responses was obtained when the concentrations of nicotine or WSF ranged from 200 to 1000 μg per ml. Suppression of humoral immune responses, however, was found, in one study, to be reversible and recovery of the immune responses occurred after cessation of exposure to cigarette smoke (Esber et al, 1973).

The above studies have clearly demonstrated the effect of exposure to cigarette smoke and other tobacco extracts such as nicotine and WSF of cigarette smoke on humoral immune responses in animals and shown that the degree of this effect depends upon the duration of exposure and the dose of cigarette smoke or tobacco extracts.

Effect of cigarette smoke on cell-mediated immunity

The effect of exposure to cigarette smoke on cell-mediated immunity (CMI) has been studied in mice and rabbit. CMI was studied *in vitro* by measuring the responsiveness of lymphocytes, from various organs of mice daily exposed to cigarette smoke for up to 35 weeks, to phytohaemagglutinin (PHA) (Thomas et al, 1973). After five weeks, the responsiveness of lymphocytes from the regional lymph nodes showed an initial enhancement. After 35 weeks exposure to cigarette smoke, however, the responsiveness to PHA of lymphocytes from these nodes was found to be markedly depressed. In contrast, the responsiveness to PHA of lymphocytes from blood and spleen was found to be depressed after 5 weeks. After 35 weeks exposure, the lymphocytes from blood showed significant decrease in the responses while lymphocytes from spleen showed no significant difference in the responses to PHA. Responsiveness was compared between mice exposed to cigarette smoke and control animals. Similar results were obtained when the reactivity of tumour-antigen specific T lymphocytes were studied in smoke-exposed mice after inoculation with viable tumour cells (Chalmer et al, 1975).

In another study (Yamakido et al, 1984), the responsiveness of lymphocytes from spleen of mice intraperitoneally injected daily with WSF of cigarette smoke to PHA and Concanavalin A (Con A) was found to be significantly depressed 8 weeks after injection. In this study, the activity of natural killer

(NK) cells was determined in another group of mice intraperitoneally injected with 5 mg of tobacco tar twice a week and was found to be decreased.

The effects of nicotine and WSF of cigarette smoke on CMI were studied *in vitro* using peripheral lymphocytes from rabbit, Con A as a T cell stimulant and goat anti-rabbit Fab (anti-Fab) as a B cells stimulant (Roszman et al, 1975). In this study, nicotine and WSF were found to suppress the mitogen-induced responses of both T cells and B cells.

Although HT and LT cigarettes have been shown to have different effects on humoral immune responses, they have been found to produce similar effects on CMI (Holt et al, 1976). In the above study, CMI against bacteria and tumour-specific antigens showed similar depression in animals exposed to HT or LT.

Effect of cigarette smoke on alveolar macrophages

In vitro studies on the effect of short-term exposure to cigarette smoke on pulmonary alveolar macrophages (PAMs) showed that exposure to cigarette smoke resulted in an immediate decrease in the viability of PAMs associated with an increase in the rate of production of (radiolabelled) RNA in the surviving cells (Holt and Keast, 1973). In this study, a significant number of PAMs lavaged from murine lungs were found to be dead and the rate of

synthesis of tritriated RNA was found to be increased 30 minutes after exposure to cigarette smoke.

In guinea pig, short-term exposure to cigarette smoke resulted in reduction of the number of PAMs (Rylander, 1971). In another experiment, short-term exposure of guinea pigs to cigarette smoke resulted in a decrease in the number of PAMs as well as in the number of leukocytes (Rylander, 1973).

Long-term exposure of mice to cigarette smoke, on the other hand, was shown to produce biphasic changes in the number of PAMs. In one study *in vivo*, the number of PAMs recovered from endobronchial lavage of mice exposed to cigarette smoke daily for up to 34 weeks showed an initial decrease in the first two weeks followed by a significant increase within 2-4 weeks (Holt and Keast, 1973).

In guinea pig, long-term exposure to cigarette smoke resulted in similar findings (Rylander, 1971). In another study, the number of PAMs and polymorphonuclear leukocytes, lavaged from the lungs of guinea pig exposed to cigarette smoke daily for up to 8 weeks, were found to be increased (Rylander, 1974).

Effect of cigarette smoke on the animal resistance to a challenge

The defence of the lungs against inhaled bacteria is achieved by the mucociliary apparatus of the upper respiratory tract and the alveolar macrophages of the lower respiratory tract (Thomas, 1967). In mice, studies indicated that clearance of bacteria from bronchopulmonary tree is an active process involving primarily the phagocytic activity of the alveolar macrophages and assisted by the mucociliary system and is not due to natural death of the organisms (Laurenzi et al, 1963; Green and Kass, 1964). In the former study, cigarette smoke was found to be one of the most pulmonary irritants of various substances tested and caused most interference with the antibacterial mechanisms of the bronchopulmonary tree.

Studies on the effect of exposure to cigarette smoke for various periods of time on the ability of the bronchopulmonary system to dispose of foreign bodies showed that both short- and long-term exposure to cigarette smoke reduce the antibacterial capacity of the lungs of animals (Rylander, 1969; Rylander, 1971). In one study, a single exposure of mice to cigarette smoke for 1, 3, or 6 hours decreased the resistance of mice to respiratory infection by *Klebsiella pneumoniae* as demonstrated by the increased mortality and decreased survival time (Spurgash et al, 1968). In this study, increasing the time interval between exposure and challenge from 1 to 48 hours resulted in decrease in the mortality

and increase in the survival time indicating that the debilitating effect of cigarette smoke is reversible.

In vitro, the phagocytic activity of rabbit alveolar macrophages against *Staphylococcus albus* was studied and found to be inhibited after exposure to cigarette smoke. This inhibition was found to vary according to the dose of cigarette smoke (Green and Carolin, 1967). In this experiment, PAMs exposed to cigarette smoke failed to adhere to the surface of the flask used in the experiment suggesting that paralysis of PAMs might be one of the mechanisms by which cigarette smoke produce its harmful effect on the respiratory system. Indeed, one study by Dalham (1966) indicated that the decrease in the bacterial clearance of the respiratory system after exposure of animals to cigarette smoke is due to paralysis of the muco-ciliary apparatus. The paralysis of the muco-ciliary apparatus in this study, however, was found to be reversible.

Cigarette smoke and tumour growth in animals

Tobacco has been shown to contain many carcinogenic substances (US. Department of Health Education and Welfare, 1971). When tobacco is burnt, tar is released and many other carcinogenic substances such as methylchlanthrene, benzopyrene and benzanthracene can be isolated (Kleinsasser, 1983). These carcinogenic substances are known as polycyclic

aromatic hydrocarbons (PAH). Inhalation of cigarette smoke in the experimental animals enhanced the growth of an established malignant cells (Thomas and Holt, 1974). In this study, only mice chronically exposed to cigarette smoke died with tumour cells in the lungs whereas short-term exposure of mice to cigarette smoke resulted in no significant difference in the tumour cell growth from that in the control group.

Therefore, the above studies in experimental animals have indicated that exposure to cigarette smoke may result in characteristic pattern of immunological and inflammatory changes. Bacterial clearance from the respiratory system and resistance to bacterial infection have been shown to be affected. Furthermore, chronic exposure to cigarette smoke have been shown to enhance growth of malignant tumours. The above findings in experimental animals have provided the impetus for further studies in humans with the possibility that similar immunological and inflammatory findings may occur in man.

Studies in man

Studies on peripheral blood

Cellular components of the blood. *In vitro* studies on human leukocytes showed that nicotine, at levels comparable to that encountered in the blood of smokers, caused a slight, although significant, depression of phytohaemagglutinin (PHA)-stimulated deoxyribonucleic acid synthesis in human leukocytes (Neher, 1974). *In vivo* studies showed that smoking results in "smokers leukocytosis" with an increase in all major classes of peripheral blood leukocytes (Core et al, 1971). Polymorphonuclear leukocytes were found to be increased up to 44% in the circulation of smokers (Bridges et al, 1985). However, the chemotactic and bactericidal activity of the polymorphonuclear leukocytes were found to be normal (Noble and Penny, 1975).

The number of monocytes in the peripheral blood of smokers was also found to be increased (Bridges et al, 1985). However, the intracellular capacity of these monocytes to kill *Candida* fungi was found to be partially defective (Nielsen, 1985). Likewise, the number of circulating basophils in the peripheral blood of smokers was found to be decreased significantly during smoking and, after abstinence for 12 hours, to rise to higher numbers than in non-smokers (Walter, 1982). The level of activity of NK cells in the circulation of smokers

was examined in two different studies (Ferson et al, 1979; Hughes et al, 1985) and was found to be depressed .

Not surprisingly, the effects of smoking on T cells populations, as one of the most important components of the immune system, has attracted much interest. Studies showed that the total number of T cells in the peripheral blood of smokers is increased (Burton et al, 1983; Robertson et al, 1983). Both total ($CD3^+$) and T helper ($CD4^+$) T cells were found to be increased in light and moderate smokers (Hughes et al, 1985). However, in heavy smokers, T suppressor/cytotoxic ($CD8^+$) T cells were found to be increased with a relative decrease in $CD4^+$ T cells (Ginns et al, 1982).

Non-cellular components of the blood. The concentrations of IgA, IgM, and IgG in the serum of smokers were reported to be reduced by up to 10-20% compared to non-smokers (Gulsvik and Fagerhole, 1979; Gerrard et al, 1980; Anderson et al, 1982). Smoking seems to produce biphasic changes in the concentration of IgE and IgD in the serum of smokers. The levels of IgE and IgD in light smokers were found to be significantly increased, whereas in heavy smokers these levels were found to be below the levels of non-smokers (Bonini, 1982; Bahna et al, 1980; Bahna et al, 1983a; Bahna et al, 1983b).

Smokers also exhibit a number of non-specific serological abnormalities compared to non-smokers. For instance, serum of smokers has been found to

contain an increased levels of C-reactive protein compared to non-smokers (Heiskell et al, 1962). Furthermore, autoantibodies such as antinuclear and rheumatoid factors were reported to be higher in smokers compared to non-smokers (Mathews et al, 1973).

Studies on the lungs

Cellular components of the bronchoalveolar lavage. Bronchoalveolar lavage fluids obtained from smokers showed characteristic features regarding alveolar macrophages. Smoking produces marked increase in the number of alveolar macrophages together with an increase in the number of neutrophils (Hunninghake et al, 1979; Harris et al, 1970; Weinberger et al, 1978). Furthermore, it seems that smoking results in activation of the alveolar macrophages and produces an increase in the metabolic activity (Harris et al, 1970), adherence (Mann et al, 1971), activity of the lysosome enzymes (Martin, 1973), and the responsiveness to chemotactic stimulants (Warr and Martin, 1974; Richards et al, 1984). However, antigen presentation and activation of T lymphocytes by alveolar macrophages was found to be depressed by smoking (Clerici et al, 1984; Lawrence et al, 1983).

The effect of smoking on the phagocytic activity of the alveolar macrophages is controversial and the various studies carried out in this respect have shown

discrepant results. While some studies showed that the phagocytic activity of alveolar macrophages of smokers is normal (Harris et al, 1970; Cohen and Cline, 1971). Other studies showed that it is deficient (Martin and Warr, 1977; Fisher et al, 1982). Presence of large numbers of alveolar macrophages was suggested to be the reason behind the depressed phagocytic activity and the suppressive effect on proliferation of T cells in the lungs of smokers (Holt, 1986).

Immunoglobulins in bronchoalveolar lavage. Generally, the IgA concentration in the bronchoalveolar lavage from smokers was found to be normal (Warr et al, 1977). The concentration of IgG was also found to be normal in some studies (Warr et al, 1977; Low et al, 1978), but was found to be increased in other studies (Reynolds and Newball, 1974; Bell et al, 1981).

Studies on saliva

The few studies which have been carried out on the effect of smoking on the immunoglobulin concentration in saliva showed discrepant results. In one study, the use of whole saliva to study the immunoglobulin concentration in smokers and non-smokers showed no difference between IgA concentration of smokers and non-smokers (Olson et al, 1985). In another study, salivary IgA concentration was found to be higher in the smokers than in non-smokers

(Watanabe et al, 1983). Other studies, however, showed that salivary IgA levels are lower in smokers than in non-smokers (Bennet and Reade, 1982; Jedrychowski et al, 1980). When pure parotid saliva from groups of healthy smokers and non-smokers was used, the concentration of IgA was found to be reduced among the smokers compared to non-smokers (Barton et al, 1990). Since whole saliva is mixed with crevicular fluids and similar in its immunoglobulin contents to serum, its use, rather than pure saliva, may produce inaccurate results and contribute to the discrepancy in the results of various studies on salivary IgA in smokers and non-smokers.

Studies on the IgG concentration in the saliva of smokers and non-smokers also produced conflicting results. In one study, IgG levels of smokers were found to be lower than in non-smokers (Jedrychowski et al, 1980). In another study, however, there was no difference in concentration of IgG of smokers and non-smokers (Barton et al, 1990). In the above study, the concentration of salivary IgM was reported to be increased in the smokers as compared to non-smokers.

Studies on cervical mucosa

The effect of cigarette smoking on the local immunity of the cervix was examined and it was found that cigarette smoking is associated with a

significant decrease in the number of Langerhan's cells, the most important antigen presenting cells in the normal cervical epithelium (Barton et al, 1988).

It was postulated that smoking is a cofactor that interact with the sexually-transmitted human papilloma virus (HPV) that results in an increase in the likelihood of developing a virally induced neoplastic changes in the cervix (Lur Hansen, 1982; Pfister, 1987). Neoplastic transformation in the cervical epithelium was found to be associated with prolonged infection with HPV (Singer and McCane, 1986). Therefore, the reduction in the number of Langerhan's cells may impair presentation of the viral antigen to T lymphocytes resulting in suppression of the local immunity of the cervical epithelium, allowing establishment and persistence of a local viral infection with likelihood of development of cervical carcinoma.

Smoking and inflammatory bowel disease

Cigarette smoking has received considerable attention in recent years as a possible factor in the pathogenesis of inflammatory bowel disease (IBD). There is increasing evidence that patients with ulcerative colitis tend to be non-smokers or ex-smokers whereas patients with Crohn's disease tend to be smokers (Bures et al, 1982; De Castella, 1982; Somerville et al, 1984). Furthermore, ex-smokers were found to be at a greater risk of ulcerative colitis

than either those who have never smoked or current smokers (Boyko et al, 1987). The above findings have led to the suggestion that smoking has a protective effect in ulcerative colitis and may be a causal factor regarding Crohn's disease.

In spite of the increasing number of epidemiological studies investigating the relationship between smoking and IBD, there have been few studies investigating this relationship in terms of the mucosal immune system. Although the exact mechanism (or mechanisms) by which smoking may influence the IBD is (are) still unknown, studies carried out in this field have postulated some possible mechanism(s) which are discussed below.

The colonic mucus, an essential component of the intestinal mucosal barrier, has been shown to be quantitatively and qualitatively abnormal in ulcerative colitis patients (Podolsky and Isselbacher, 1984). Cigarette smoking has been found to produce hypersecretion and modification of respiratory mucus by systemic and local effects (Kollerstrom et al, 1977). *In vitro* study of colonic mucus production has shown that patients with ulcerative colitis have reduced mucus production compared with controls, and that ulcerative colitis patients who did not smoke showed reduced mucus production compared with non-smokers control (Cope et al, 1986). Therefore, the increased production of colonic mucus found in smokers may improve the quality of the mucosal

barrier and may explain how cigarette smoking may protect against ulcerative colitis.

The lining epithelium of the intestinal mucosa is another important factor involved in the intestinal mucosal defence system. The integrity and effectiveness of this barrier could be assessed by measuring the urinary recovery of orally administered 'probe' molecules such as ^{51}Cr-EDTA (which does not cross through intact epithelium). It has been found that 24 hour urinary recovery of ^{51}Cr-EDTA is significantly lower in healthy smokers compared to healthy non-smokers suggesting that smoking may tighten the intestinal epithelium by reducing the paracellular junctions or by decreasing the permeability of the intestinal epithelium (Prytz et al, 1989).

Smoking may also influence the blood flow in the rectum. When rectal blood flow was measured using laser Doppler flowmetry, patients with ulcerative colitis in remission (all non-smokers) were found to have increased rectal blood flow compared with healthy smokers and non-smokers controls (Srivastava et al, 1990). In this study, acute cigarette smoking resulted in a pronounced decrease in the rectal blood flow in all subjects but patients with colitis had significantly higher values compared with smokers. The mechanism which has been suggested to be involved in the reduction of rectal blood flow during smoking is nicotine-induced vasoconstriction and the reduction in blood flow

may decrease the amount of inflammatory mediators reaching the mucosal surface decreasing the likelihood of developing ulcerative colitis.

Inflammatory mediators such as prostaglandin E_2 (PGE_2) and leukotriene B_4 are known to be increased in IBD. These mediators are also known as eicosanoids. In the gut, cigarette smoking was found to reduce both luminal and tissue PGE_2 in the gastric mucosa (McCready et al, 1985; Quimby et al, 1986). In one *in vitro* study, the production of eicosanoids by the colonic mucosa of cigarette smokers was found to be lower than that of non-smokers (Motley et al, 1990). In another *in vitro* study, the colonic production of PGE_2 in patients with ulcerative colitis was found to be significantly decreased in heavy smokers compared to those who have never smoked (Cope et al, 1991).

Therefore, the above findings suggest that smoking may have an inhibitory effect on the release of the inflammatory mediators rendering the colonic mucosa less liable to the inflammatory process and development of ulcerative colitis.

Immunoglobulin production in the gut may also be affected by smoking. IBD is known to be associated with altered immunoglobulin production. *In vitro* studies on the immunoglobulin production by the colonic mucosa in IBD patients and controls showed that smoking decreases the production of IgA in both IBD patients and controls, markedly decreases the production of IgM in

IBD patients and increases the production of IgG in the smokers (Cope et al, 1989). This study suggests that smoking may be involved in the pathogenesis of IBD by suppressing the local intestinal immunity. However, one *in vivo* study, using whole gut lavage as a material for the investigation, found that there was no significant difference between the concentration of intestinal IgA, IgM and IgG in healthy smokers and non-smokers (Srivastava et al, 1991). This study also showed that there was no significant difference between smokers and non-smokers with ulcerative colitis. Therefore, the results of the latter study cast doubts on the suggestion that smoking may influence the pathogenesis of IBD by interfering with the normal humoral mucosal immune system.

Finally, the mechanism (or mechanisms) by which smoking may interfere in pathogenesis of IBD (and may be other immune disorders in the gut) is/are still, as yet, not well understood. Obviously, laboratory investigations, particularly in terms of intestinal mucosal immunity, are required to support the findings of the epidemiological studies to answer the question: does smoking have any role in the pathogenesis of the immune disorders of the gut ?

Therefore, WGL, as a non-invasive, safe and useful method to obtain intestinal secretions can be used to investigate the effect of smoking on the immunity. The use of reliable method which provides a direct access into the intestinal secretions may help in explanation not only the inflammatory changes associated with cigarette smoking but may also help in explaining some of the

neoplastic complications related to smoking. Furthermore, the use of reliable methods to investigate the effect of smoking on the intestinal immune system may help in moving from the stage of reporting immunological changes associated with smoking to the stage where such immunological changes can be correlated with the occurrence of smoking-related diseases.

Chapter III

MATERIALS AND METHODS

Section 1

WHOLE GUT LAVAGE: PERSPECTIVE VIEW AND TECHNICAL DEVELOPMENT

Introduction

In humans, a non-invasive technique that can provide a direct access into intestinal secretions has always been the aim of many investigators. Elson and colleagues (1984) reported a "relatively easy" method by which a lavage solution could be exploited to obtain repeated samples of intestinal secretions from mice, and the possibility of measuring both total IgA and antigen-specific antibodies in these secretions. A modification of the above method was reported by Gaspari et al (1988) who described a whole gut lavage technique in humans for obtaining intestinal secretions using a commercially available lavage solution (Golytely). The process of collecting, processing and analysing human intestinal secretions was proved to be feasible and whole gut lavage fluids were found to be useful material for a variety of investigations on gut immunity. O'Mahony and colleagues (1990) published their preliminary study on the use of whole gut lavage technique in patients with coeliac disease or Crohn's disease. In the above study, total immunoglobulins and antigen-specific antibodies in intestinal secretions of the patients studied were readily measured by ELISA and whole gut lavage technique was found to be safe and well-tolerated by most of the patients involved in the study.

I have further evaluated the use of whole gut lavage for obtaining intestinal secretions to study gut immunity in healthy British volunteers, patients with a variety of gastrointestinal diseases as well as patients who had had salmonella infection. A sequential lavage study was carried out in another group of patients to validate the use of any clear specimen passed per rectum, during the whole gut lavage process, as a representative of the rest of whole gut lavage effluent.

The lavage solution

The commercial lavage solution used in whole gut lavage has essentially been used for cleansing the colon as a preparation for Barium enema (Skucas et al, 1976), colonoscopy (Rhode et al, 1977), colon surgery (Hewitt et al, 1973) as well as for the treatment of intractable constipation (Puxty and Fox, 1986). The main constituent of the lavage solution is polyethylene glycol (PEG) which has a molecular weight of 3350 and used at a concentration of 59 g/litre tap water. PEG is a non-absorbable material that produces an osmotic diarrhoea and does not cause net absorption or secretion of fluids or electrolytes across the wall of the intestine (Davis et al, 1980; Malawer and Powell, 1976). Other contents of the lavage solution include sodium chloride (BP 1.45g), sodium bicarbonate (BP 1.63g), sodium bisulphate (BP 5.68g) and potassium chloride (BP 0.75g).

The lavage solution has now been marketed as "Golytely" or "Klean.prep" and is available in small sachets to be made up to one litre by adding tap water resulting in an osmolality of 260 mosm/litre.

Lavage protocol

The approval of the Medicine and Clinical Oncology Ethics of Medical Research Subcommittee of Lothian Health Board was obtained before commencing the study of whole gut lavage (WGL) and written consent was obtained from every participant. Subjects involved in the study were asked to fast overnight and to come next morning to the G.I.Unit to start drinking the lavage solution at about 08.30. Before subjects started drinking the lavage solution, a brief medical history was recorded and 10-20 mls of venous blood samples were taken from every participant. Because lavage solution was found to be more palatable if consumed chilled, it was made up and kept in the fridge for a few minutes before it was consumed. Subjects were asked to drink the lavage solution at a rate of 200 mls every 10-15 minutes for a period of 3-4 hours till the total volume consumed was 3-4 litres i.e. at a rate of one litre per hour. Subjects were also asked to record the time at which they started and completed each glass of the lavage solution and to stop drinking at any time should they find the procedure intolerable.

Within 2 hours, the bowel of the subjects started to move. Solid and semi-liquid stools were discarded and only clear fluid stools passed per rectum were collected. Once the subjects started to pass clear fluid stools per rectum, they were allowed to have a cup of tea or coffee with some biscuits and to leave.

Clear fluid stools were collected in 2-3 20 mls universal containers and sent to the gastrointestinal (GI) laboratory within 10 minutes of collection where protease enzyme inhibitors were added to the lavage specimens, to minimise degradation of proteins by protease enzymes present in large amount in the intestinal secretions i.e. to be processed (see below), and stored. For the purpose of measuring the PEG contents in the stool samples, a specimen of the lavage solution consumed by the subjects was collected in one to two 20 mls universal containers before or during the experiment.

Blood samples were divided into two small 10 mls tubes, one of them was sent to the Department of Clinical Biochemistry for the assay of total immunoglobulin concentration whereas the other tube was sent to the G.I laboratory for serum extraction and storage.

Processing of specimens

Once clear fluid stools were obtained from subjects involved in the WGL study, specimens were transported immediately to the G.I laboratory for processing and storage. This is because a previous study (O'Mahony et al, 1990) has shown that a delay of 1-2 hours in processing lavage stools may result in up to 92% loss in IgA contents of the specimens.

Specimens were filtered through glass filter papers (Whatmann Glass Microfibre Filters 12.5 cm) into 50 mls test tubes. Before adding protease inhibitors, approximately 3 mls of the filtered fluid was dispensed into 2 x 1.5 mls tubes and 20 µl of sodium azide solution (20 g/litre distilled water) was added to each tube to be used for the measurement of protease enzymes activity in the processed and unprocessed lavage specimens (see below).

To a 20 ml universal container, 10 mls of the filtered lavage fluid was transferred and 1 ml of Soybean trypsin inhibitor (SBTI) (Sigma), at a concentration of 1 mg/ml in phosphate buffer solution (PBS), was added. After mixing, 0.56 ml of 0.1M ethylenediaminetetracetic acid (EDTA) (as a chelating agent for calcium and magnesium ions which are required for the activation of protease enzymes) was added. After mixing, 0.24 ml of 0.1M in 95% alcohol phenylmethylsulphonyl fluoride (PMSF) was added and mixed. Thereafter, 0.12 ml of sodium azide (bactericidal agent) (20 g/litre distilled

water) was added. After leaving the mixture to stand for two minutes to allow protease enzyme inhibitors to work, 0.6 ml of heat-inactivated new born calf serum (NBCS) was added to the mixture to provide an alternative substrate for any remaining protease enzymes. The final volume of the mixture in the universal container was 12.5 mls. The mixture was then divided into 8 aliquots dispensed in 8 x 1.5 tubes and stored at -70 °C for analysis later in various experiments.

Assays performed on WGL fluid

The following assays were performed on processed WGL fluids. Details of the methodology will be described later in this chapter.

Total and antigen-specific immunoglobulin concentration assay: Enzyme-linked immunosorbent assay (ELISA) was performed to measure total immunoglobulins (Ig) A, M and G, secretory IgA, anti-*Salmonella typhi* lipopolysaccharide (LPS) antibodies and anti-*Salmonella typhimurium* RC-mutant antibodies in processed WGL fluid.

Polyethylene glycol (PEG) assay: The concentration of PEG was measured in processed lavage samples to examine the degree of dilution of the intestinal secretions by the lavage solution. In a sequential lavage study, PEG

concentration (in addition to many other indices in the lavage fluid effluent) was measured in 5-6 serial lavage specimens obtained from every subject involved in the study to investigate the validity of taking one clear specimen of the lavage effluent as a representative of the rest of the effluent.

Protease enzymes activity assay: The effect of adding protease enzymes inhibitors and the degree of inhibition was studied in both processed and unprocessed lavage fluid.

Albumin concentration in WGL fluid assay: Excessive amounts of albumin in WGL fluid may indicate the presence of an abnormal section of the gut, e.g. an ulcer, that allows leakage of albumin molecules from the systemic circulation into the gut lumen.

Alpha-1-antitrypsin in WGL fluid assay: The amount of alpha-1-antitrypsin in WGL fluid may be another indication of the amount of the protein loss across the gut due to an inflammatory process in the mucosa of the gut.

Subjects studied

Twenty-two healthy British volunteers were recruited for the study of the effect of the newly licensed oral typhoid vaccine Ty21a on the intestinal immune

status of individuals from areas which are known to be non-endemic with typhoid fever such as the United Kingdom (UK). This group of volunteers was further divided into 14 heavy smokers and 8 non-smokers to investigate the influence of heavy smoking on gut immunity and the effect of boosting the immune status of these smokers/non-smokers subjects with a mitogenic substance such as the oral typhoid vaccine Ty21a.

For the sequential lavage study, 11 patients were recruited from the out-patient clinic as well as from the in-patient department of the gastrointestinal unit (GIU) of the Western General Hospital. These are patients who were attending the GIU to be treated from a variety of G.I diseases or patients who were admitted to be prepared for lower endoscopy (flexible sigmoidoscopy and/or colonoscopy).

To study the effect of naturally acquired salmonella infection on the gut immunity, a group of 8 patients were recruited from the City Hospital after treatment for salmonella infection. These were mostly patients who had returned from holidays abroad and were referred to Dr. Gray of the City Hospital with manifestations of salmonella infection.

To investigate the phenomenon of intestinal IgA deficiency in subjects with normal concentration of serum IgA I observed while investigating the immunoglobulin contents in WGL fluid of patients with a variety of

gastrointestinal diseases, I retrospectively studied WGL specimens obtained from normal individuals as well as from patients attending the GIU for a variety of gastrointestinal diseases during 1991 and 1992. These specimens were stored at -70 °C.

WGL specimens of patients with inflammatory bowel diseases (IBD) obtained during 1991 and 1992 were studied with respect to the total immunoglobulins and antigen-specific antibody concentrations in these specimens, comparing them with the findings in lavage specimens obtained from healthy volunteers. Further details of all subjects (healthy volunteers and patients) involved in the different studies cited in this thesis will be described in the relevant chapters.

Section 2

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Introduction

During the last three decades there has been a prominent increase in the number and variety of methods available for immunodiagnostic tests. This is because there has been marked development in the methods which use labelled antigens or antibodies resulting in more sensitive and specific tests.

Radioimmunoassay (RIA) was regarded as the method of choice for assays of both large and small molecular weight substances (Sonksen, 1974) and immunofluorescence was found to be a useful method for the measurement of antibody levels in infectious and auto-immune diseases (Nairn, 1976). However, both tests have their limitation. RIA requires rather sophisticated and expensive equipment, the reagents utilised in have short shelf-life and the strict regulatory controls on the use of isotopes have tended to exclude RIA from many small laboratories. Immunofluorescence was found to be tedious, time-consuming and can be used for only small batches of tests (Voller et al, 1978). Old tests based on haemagglutination and complement fixation were found to be less accurate than modern assays such as RIA. Therefore, the search for alternative methods which use other labels for antigens and antibodies has continued and ELISA was found to be the most promising method which uses

enzymes that can be linked to antigens and antibodies and the complexes produced have both immunological and enzymatic activity.

ELISA was first described in the early seventies (Engvall and Perlmann, 1971) and is nowadays regarded as at least one of the most sensitive and specific methods for detecting both antibodies and antigens and has rapidly superseded other methods for the quantitative measurement of antigen-specific antibodies as it can be used for large-scale tests and it is relatively economical in the use of reagents required for the tests. Furthermore, reagents used in ELISA have long shelf-life and there are no radioactivity hazards.

There are two main types of ELISA techniques, homogeneous and heterogeneous. Homogeneous method involves the simultaneous use of all reagents in one step. It is particularly useful for rapid assays of low molecular weight compounds such as drugs (e.g. morphine, digoxin and gentamicin) and some hormones (e.g. T3, T4 and cortisol). However, homogeneous methods are generally not suitable for detecting larger molecules such as microbial antigens and their antibodies.

The heterogeneous type of ELISA can be further classified into competitive and non-competitive ELISA. The latter technique was the main type of ELISAs used in the research work described in this thesis where it was used for detection of total immunoglobulins, using Double Antibody Sandwich

technique, and antigen-specific antibodies, using the indirect method of the non-competitive ELISA.

Measurement of total immunoglobulins by ELISA

The method most commonly used for antigen detection and quantitation is the double antibody sandwich (non-competitive) ELISA. Double antibody sandwich involves the following steps:

1. ELISA plates, a 96-well microtitration plastic plates (the solid phase) is coated with specific antibodies (usually animal derived antibodies) and incubated overnight. For the measurement of TIgA, 96-well Immulon1 129A (Dynatech) ELISA plates were coated with 200 ng/ml affinity-purified goat anti-human IgA antibodies in 0.05M carbonate buffer, pH 9.6, (Northeast Biomedicals). Coating antibody and all reactants in the following steps were added in volumes of 0.125 mls per well. Coated plates were incubated overnight at 4 °C.

2. Following overnight incubation, free antibodies are washed away. Plates were then blocked with low molecular protein such as bovine serum albumin (BSA) or adult bovine serum to minimise any non-specific binding. For the assay of TIgA, wash solution was composed of 0.05%

Tween 20 (Sigma) added to 1 litre normal saline. Tween 20, a detergent, was added to minimise non-specific reactions. Plates were washed three times either manually using multi-channel pipette or automatically using ELISA-wash machine (Wellwash). The solution used for blocking was composed of 0.05% Tween 20, 1% adult bovine serum (Span) added to 1 litre normal saline.

3. Standard and test samples are added to the coated plates at two-fold serial dilutions and at an appropriate initial dilution of the standard and test samples and then incubated. Human colostrum with an initial dilution of 1.25 μg/ml of IgA was used as a standard in the assay performed for the measurement of TIgA concentration in WGL fluid, saliva and jejunal aspirate. The initial dilution of the test samples was 1/100. The solution used for dilution of the standard and test samples (ELISA diluent) was composed of 0.05% Tween 20, 1% adult bovine serum in 1 litre normal saline. Plates were then incubated overnight at 4 ^{o}C.

4. Unbound proteins are washed away and an enzyme-labelled specific antibodies (conjugate) is added at an appropriate concentration to every well. The two most commonly used enzyme labels are alkaline phosphatase and horseradish peroxidase. Goat anti-human IgA labelled with alkaline phosphatase (Northeast Biomedicals or Sigma) at a concentration of 1/5000

in ELISA diluent was used as a conjugate for the measurement of TIgA in WGL fluid. Plates were then incubated for 3 hours at room temperature.

5. Free conjugate is washed away while the bound conjugate is visualised by the addition of the substrate. The substrate is a chromogen substance which is degraded by the enzyme of the conjugate to produce a stable, soluble, and coloured end-product (Voller et al, 1978). There are many types of substrates and the choice of the appropriate one is governed by the enzyme used. Para-nitrophenyl phosphate (PNP) is the only colorometric substrate that is used with alkaline phosphatase conjugate and found to be safe and available in tablet form that produces a stable yellow end-product. The quantity of the test antigen is assessed by the change in the colour of the end-product i.e. optical density of the test solution using a spectrophotometer. For the TIgA assay, PNP (Sigma) 1 mg/ml in 10% Diethanolamine (DEA) buffer at pH 9.8 was added to every well and the plates were read using ELISA reader (MR 5000, Dynatech) at wavelength of 405 nm. When the optical density of the standard was 1.000 and at least 3 of the test samples dilutions were within the range of the standard, the test was technically completed.

Interpretation of the results

The concentration of the antigen in the sample is measured by reference to a standard curve prepared by plotting the optical density (OD) values of serial dilutions of a standard containing known concentration of the antigen being measured. This involves plotting a curve of the OD on Y-axis against the concentration of the standard on X-axis. Immunoglobulin concentration in the test samples are then measured by comparing the OD of the test sample with that of the standard. i.e. extrapolating the immunoglobulin levels of the test samples from the standard curve.

For TIgA measurement, a standard curve was constructed by plotting the OD of the standard dilutions on the Y-axis against the log-transformed values of the concentration of the standard on the X-axis resulting in a straight line. The standard solution (human colostrum) contained known amount of the TIgA (mostly secretory IgA) starting from 1250 ng/ml decreasing to 19.350 ng/ml. The correlation coefficient (r) of the standard curve was obtained and the technique was regarded a satisfactory one only when r value of 0.99 could be obtained. The concentration of TIgA in the test samples was determined by taking the mean value of 2-3 test samples calculated from the OD values of these samples when they fell within the range of the standard curve (Fig 3.I).

ELISA used for the measurement of TIgM and TIgG levels in lavage samples were basically similar to the above described method. Human reference serum (Protein Reference Unit PRU, Sheffield) was used as a standard with an initial dilution of 1000 ng/ml. The initial dilution of the test samples was 1/25 for IgM and 1/10 for IgG. Results were read and interpreted as described for IgA.

Measurement of secretory IgA concentrations in WGL fluid

The ELISA technique used for the measurement of secretory IgA (SIgA) levels in WGL fluid was basically similar to the ELISA performed for the measurement of TIgA concentration, with the exception that the conjugate used for TIgA assay (goat anti-human IgA alkaline phosphatase) was replaced by conjugate that is specific for SIgA. This conjugate is called anti-human IgA secretory piece alkaline phosphatase (The Binding Site Ltd.). The conjugate used for SIgA assay was added to the well at a dilution of 1/2000 in ELISA diluent. The same standard, human colostrum IgA, of which SIgA is almost 100%, was used for SIgA assay.

Measurement of antigen-specific antibodies in WGL fluid

ELISA has been used with enthusiasm by workers on infectious diseases where the need is often for the measurement of antibodies against a variety of infectious agents. Investigators have found that the indirect method of ELISA is particularly suitable for the measurement of antigen-specific antibodies titres (Engvall and Perlmann, 1972).

The indirect method of ELISA involves immobilisation of the antigen by passive adsorption on to the solid phase. Test samples are then incubated with the solid phase and any antibodies in the test samples become attached to the antigen. After washing to remove unreacted components of the test samples, an anti-globulin enzyme conjugate is added and incubated. This will become attached to any antibodies bound to the antigen. Washing again will remove unbound reactants and finally the substrate is added. The colour change of the substrate will be a measure of the conjugate fixed which itself is proportional to the antibody levels in the test samples.

The actual concentration of the specific antibodies in the substance being tested is rather difficult to measure because the initial concentration of the standard is not known and it is somewhat difficult to determine, in absolute weight, the precise amount of the antigen-specific antibodies in the standard solution. Investigators, therefore, used to express the results obtained by the indirect

method as the percentage of OD of the test samples and the standard solution. An alternative way of expressing the results is the designation of an arbitrary value for the standard "X units" then a standard curve is constructed from the serial dilution of the standard. The antibody contents of the test samples is then extrapolated from the standard curve.

Measurement of anti-*Salmonella typhi* LPS antibodies

The following technique was used for the quantitative measurement of anti-*Salmonella typhi* lipopolysaccharide (LPS) antibodies in WGL fluid and serum.

1. ELISA plates (Immulon2 129B, Dynatech) were used for the measurement of anti-*Salmonella typhi* LPS antibodies in lavage specimens and serum. These plates allow the coating antigen to be adsorbed more strongly to the solid phase than Immulon1 plates. Five to ten mgs of *Salmonella typhosa* LPS (Sigma L-6386) were dissolved 1:1 (w/v) in distilled water then a calculated volume of the solution was added to carbonate buffer solution (Sigma) to give a final concentration of 1 μg/ml. Wells were coated with 100 μl of the coating antigen and incubated overnight at 4 °C. Plates were washed three times as described for TIgA then blocked for 45 minutes. The blocking buffer (as well as the diluent) was composed of 0.05% Tween 20,

0.05% (w/v) bovine serum albumin (Sigma A-7906) added to one litre normal saline. Adult bovine serum (used for ELISA block and diluent in TIgA assay) was excluded because it produced high non-specific reaction and gave inaccurate results in this assay.

2. Serum from a colleague found to have a high titre of anti-TAB vaccine (typhoid vaccine) antibodies was collected and aliquoted into small tubes which were stored at -70 °C to be used as a reference standard for this assay. The initial dilutions of the standard used for the quantitative measurement of anti-*Salmonella typhi* LPS antibodies in WGL fluid and serum were 1/100, 1/200 and 1/2000 for the measurement of IgA, IgM and IgG antibodies respectively whereas the initial dilution of the test samples were 1/2 for WGL fluid and 1/100 for serum samples. The standard solution and test samples were added in duplicate into the ELISA plates and a series of doubling dilutions were made as for the TIgA assay. Plates were then incubated overnight at 4 °C.

3. After washing, goat anti-human IgA, IgM or IgG alkaline phosphatase conjugate (Sigma Immuno Chemicals) was added at the appropriate concentration and incubated for 3-4 hours at 22 °C. The rest of the technical procedure and reading the plates was carried out as for TIgA assay.

4. Antibody titres in the test samples were extrapolated from the standard curve. An arbitrary value of 1000 units/ml anti-*Salmonella typhi* LPS antibodies were designated for the standard and the results of the test samples were expressed as units/ml. However, after obtaining the TIgA results (μg/ml) of the same samples, results were expressed as units/mg TIgA. (Fig 3.II).

Specificity of anti-*Salmonella typhi* LPS antibodies assay

Because other *Salmonella* spp, other than *Salmonella typhi*, as well as other members of Enterobacteriaceae may share some of the *Salmonella typhi* antigens, the specificity of the anti-*Salmonella* LPS antibodies assay was further investigated. LPS of a *Salmonella typhimurium* mutant (Rc-mutant) was used as a coating antigen. This antigen was prepared by Dr. McLoughlin (department of Medical Microbiology, University of Edinburgh) and the standard was prepared by collecting blood samples from 6 patients with active inflammatory bowel diseases and the serum obtained was stored at -70 °C to be used as a standard. This work was carried out by Dr. McLintock of the G.I. laboratory, Western General Hospital.

Generally, the following conditions were necessary to meet while performing all types of ELISA in order to obtain satisfactory results:

1. Optimum concentration of antibodies or antigens used for coating of the solid phase.

2. Optimum incubation time and the temperature at which ELISA plates to be incubated.

3. Optimum composition of the solution used for ELISA wash, block and diluent.

4. Optimum choice of the conjugate and substrate as well as the time required by the substrate to produce the optimum OD.

Section 3

DETERMINATION OF POLYETHYLENE GLYCOL LEVELS IN WGL FLUID

Introduction

Hydén (Hydén, 1955) described a turbidimetric analysis of polyethylene glycol (PEG) in biological material based on the development of an oil-in-water emulsion of PEG when exposed to trichloroacetic acid (TCA) in the presence of barium ions. This method was, however, found to be adversely affected by various factors which affect colloidal solutions in general e.g. temperature, pH, vibration, method of mixing, interfering ions etc.

Later on, Malawer and Powell (1967) reported the development of an improved method for PEG assay in which they introduce an emulsifying agent (gum Arabic) to stabilise the oil-in-water emulsion. In the above study, the introduction of an emulsifying agent into the PEG assay was reported to produce more accurate and reproducible results.

Procedure

The method described below is mainly based on the method reported by Malawer and Powell (1967). This method involves the use of PEG 3350 (BDH

Biochemicals) at a concentration of 1 g/100 ml in distilled water as a standard solution. Standard solution was added to four test tubes at 0.2, 0.4, 0.6, and 0.8 ml to which distilled water was added to make up 1 ml solution. This would give a solution (standard) of PEG which varies in its concentration from 200 to 800 mg/ml. One ml of distilled water was added to a fifth tube to be used as a blank.

Lavage samples and PEG solution used as standard were diluted 1/10 in distilled water and added in duplicate into 20 ml test tubes. To all tubes, the following were added (all reagents were supplied by BDH Biochemicals): 10 mls distilled water, 1 ml of 10% (w/v) $BaCl_2.2H_2O$, 2 mls $Ba(OH)_2$, and 2 mls of 5% (w/v) $ZnSO_4.7H_2O$. The tubes were then mixed vigorously for one minute and left to stand for 10 minutes. The fluids were then filtered through double thickness (Whatmann no. 42) ashless filter papers. One ml of the filtrate was added to 3 mls gum Arabic solution (10 mg per litre distilled water), mixed and then 4 mls of 30% (w/v) trichloroacetic acid (TCA) containing 5% (w/v) $BaCl_2.2H_2O$ solution was added. Tubes were then sealed with parafilm and left to stand for 75 minutes at room temperature. The optical density of the test samples and standard were read by a spectrophotometer (Pye Unicam PU 8610, Philips) at a wave length of 650 nm. A standard curve was constructed and PEG concentration of test samples were extrapolated from the standard curve.

Section 4

DETERMINATION OF ALBUMIN CONCENTRATION IN WGL FLUID

Introduction

Determination of albumin concentration in WGL fluid was based on the fact that human albumin reacts with its specific antibody forming immunocomplexes which precipitate quickly in the presence of PEG. The precipitate produces turbidity the density of which is related to the concentration of albumin in the lavage samples (immunoturbidimetric assay). The turbidity is then measured photometrically at a wave length of 340 nm and the optical density obtained is used to construct a standard curve from which the concentration of the albumin in the test samples can be extrapolated.

Reagents used in albumin assay

The reagents used were as follows.

PEG reagent. This is composed of 40g PEG 6000 (BDH Biochemicals), 6g tris buffer, 2g Tween 20 (Sigma) and 1g sodium azide dissolved in 1 litre distilled water and adjusted to pH 7.0 with HCl.

Antibody reagent. Composed of sheep anti-human albumin serum (SAPU code S034-205) diluted 1/50 in PEG reagent (to be prepared fresh on day of assay).

Diluent. Composed of 9g sodium chloride, 60g PEG 3350 and 1g sodium azide in 1 litre distilled water.

Calibration standard. Human serum SPS-01 (PRU-Sheffield) diluted to give albumin concentrations of 10, 20, 50, 100, and 200 μg/ml in diluent.

Procedure

The procedure involves diluting 50 μl of the standard solution or test samples with 0.95 ml PEG reagent into 3 ml test tubes to be used as blanks. Another 50 μl of the standard solution or test samples were added into 0.95 ml antibody reagent in 3 ml test tubes. After leaving to stand for 15-20 minutes, standard solution, test samples and blanks were read by spectrophotometer (Pye Unicam PU 8610, Philips) at a wave length of 340 nm. A standard curve based on the optical density obtained was constructed and the test samples were extrapolated from the standard curve.

Determination of alpha-1-antitrypsin in WGL fluid

The methodology used for determination of alpha-1-antitrypsin concentration in WGL fluid was based on the same principle, reagents, and procedure described above for the determination of albumin concentration in WGL.

Determination of protease enzymes activity in WGL fluid

Processed and unprocessed lavage specimens were required for this assay. A suspension of Azacol (Sigma A 9404) in 10 mg/ml phosphate buffer solution (PBS) was prepared. Half a ml of this suspension was transferred into 3 ml polystyrene test tubes which were then put in a water bath at 37 ^{o}C. The reaction was started by the addition of 50 µl lavage specimens (processed or unprocessed). The reaction was stopped after 30 minutes by transferring the test tubes to iced water for 5 minutes. Tubes were then centrifuged (using Mistral 3000i, MSE centrifuge) at 2000 rpm at 4 ^{o}C for 5 minutes and 0.1 ml of the supernatant was added to 0.9 ml PBS before mixing the tubes. The solution was then read by spectrophotometer (Pye Unicam PU 8610, Philips) at a wave length of 530 nm. The percentage of inhibition of protease activity in the processed samples were determined by calculating the optical density of the processed and unprocessed specimens.

Section 5

QUANTITATION OF IMMUNOGLOBULIN-PRODUCING CELLS IN THE LAMINA PROPRIA OF SMALL BOWEL BIOPSIES USING IMAGE ANALYSIS

Background

Image analysis may be used to identify various parameters such as size, number, shape, position, and optical density of identifiable parts of an image. Image analysis requires a microscope, video camera, visual display monitor, computer with double-disc drive and a printer. An image from the microscope is transferred by the video camera to the monitor. The image on the screen is modified to give an image suitable for measuring (the image is converted into a binary image) where different grey intensities can be detected. Detectable areas can be measured and the values can be recorded.

Procedure

The image analyser used was manufactured by Leitz (TAS plus, Bosch). Sections of intestinal biopsies were prepared and stained immunohistochemically (by Mr. Bode, MLSO technician in the G.I laboratory, WGH) using either IgA or IgM polyclonal antibodies. Sections that contained

high interstitial immunoglobulins were excluded because they attract large quantities of the stain making the section rather difficult to count. The microscope was set to Kholer illumination to obtain optimum resolution and the slides were adjusted to be examined under x100 objective. The appropriate software programme was loaded into the computer of the analyser. IgA- or IgM-containing plasma cells were counted in the lamina propria of every field examined and the area of lamina propria in which these cells were counted was measured excluding the areas of the intestinal glands such as crypts of Lieberkühn. Up to 50 different fields were examined systematically per slide. When counting was completed, a print out of the final measurements including the total number of cells counted, the total size of the area of lamina propria measured and the mean number of cells per square millimetre was obtained. Results were expressed as number of positive cells per square millimetre of lamina propria.

Section 6

MISCELLANEOUS METHODS AND MATERIALS

Oral typhoid vaccine Ty21a

This is a live attenuated mutant strain of *Salmonella typhi* Ty21a (Vivotif Berna, and Vivotif Evans, Switzerland) which is contained in enteric-coated capsules. Each capsule of the vaccine contains at least 10^9 live *Salmonella typhi* Ty21a organisms in a lyophilised form. The live organisms in the capsules have lost their pathogenicity due to irreversible change in the cell wall. The vaccine is unstable at normal room temperature and therefore the vaccine should always be stored at 4 ^{o}C. More detail about this vaccine has been described earlier in chapter 2.

Statistical methods

A data analysis software programme (Minitab, versions 7 and 8) was used to perform statistical tests on results of the various studies described in this thesis. Whenever data were found to be normally distributed, levels were expressed as means. Student "t" test was used to compare matched pairs of observations (Paired sample t-test) or independent samples (Two-sample test). Pearson

correlation coefficient (r) was used to examine the relationship between two variables.

For non-parametric data, on the other hand, levels were expressed as median and ranges. Wilcoxon signed-rank test was used for paired data whereas Mann-Whitney U-test was used for independent set of data. Spearman's rank correlation coefficient was used to study the relationship between two variables.

Computer work

IBM and IBM compatible computers were used during the research work for handling, storage and presenting data and a variety of computer software packages were employed for this purposes. For data storage, dbase III plus (Ashton-Tate Corporation) was used. Graphical representation of the results were performed using GraphPad Inplot (GraphPad software Inc. version 4) software. Word processing of the text was done using Word for Windows (Microsoft Corporation, version 6.0) and references were organised using Reference Manager (Research Information Systems, Inc. version 5.01). Statistical analysis of the data were performed using Minitab (Minitab statistical software, release 7 and 8).

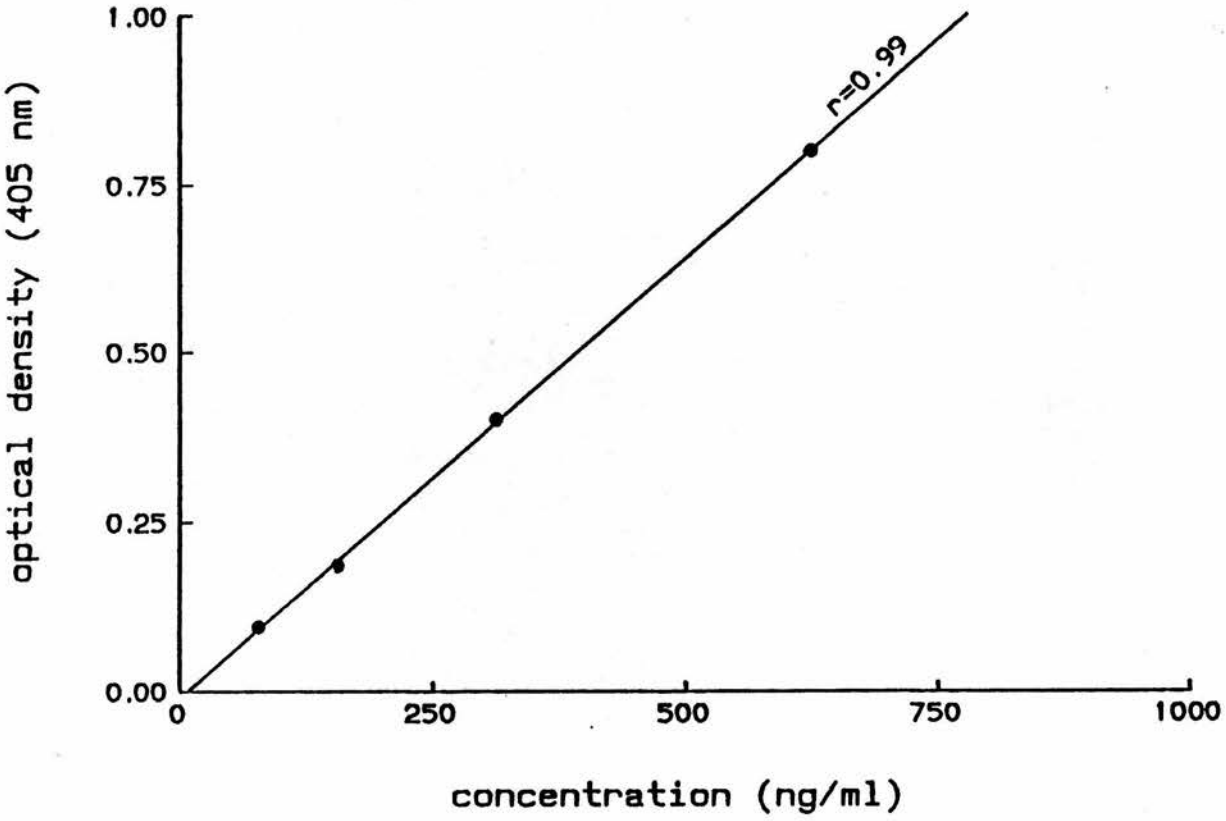


Fig 3.I. Standard curve of total IgA in WGL fluid.

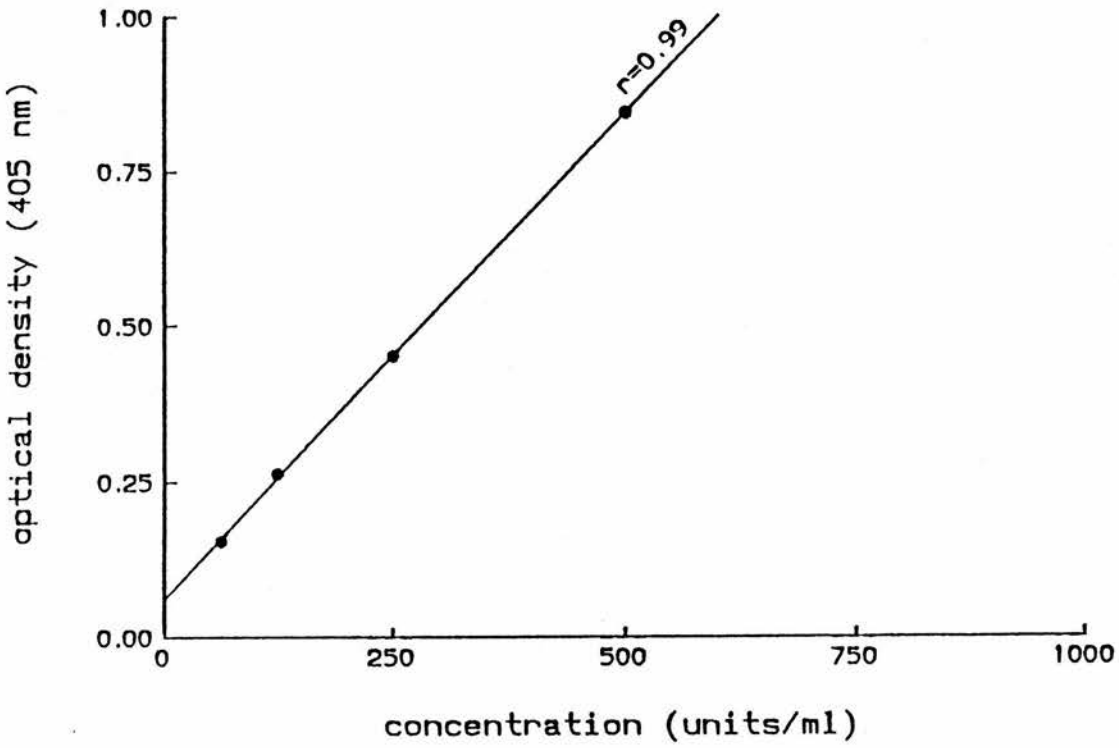


Fig. 3.II. Standard curve of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid.

Chapter IV

SERIAL LAVAGE STUDY

Introduction

As mentioned before, obtaining intestinal fluids as a material to investigate the intestinal mucosal immune system is regarded as the "gold standard" by which other methods may be compared. Elson et al (1984) reported the use of WGL technique for quantitative measurements of intestinal immunoglobulins and antigen-specific antibodies in mice followed by Gaspari et al (1988), who described the use of WGL in man. The technique was first introduced into the gastrointestinal unit of the Western General Hospital in 1988. Since then, WGL has been used to collect material to investigate the intestinal mucosal immunity in patients with a variety of gastrointestinal diseases, as well as in volunteers for various immunological studies.

Since the introduction of WGL technique, a great deal of technical modification and development have been made to the process of collecting, processing and storing the fluids obtained by WGL method. Obtaining intestinal fluids by WGL method is non-invasive and has been found to be a safe and reliable method for collection of intestinal secretions to study the local intestinal immunity.

In practice, the first clear sample of the lavage effluent is taken as representative of the rest of the lavage fluid. However, an important question has always been asked: how representative is the first clear sample

of the rest of the lavage fluid ? Although O'Mahony et al (1990) addressed this point, they only studied up to two clear serial lavage samples from every patient (n=14) and only the concentration of total IgA was measured in these serial lavage samples. Further study, using more than two clear samples from every subject and measuring other substances (such as the various immunoglobulins, antigen-specific antibodies, alpha-1-antitrypsin and other proteins and proteases activity) is obviously important to validate the use the first clear sample as a representative of the rest of the lavage fluid.

Therefore, the study of O'Mahony et al (1990) may not be sufficient to establish whether gut lavage effluent is a perfusate, in which case any clear sample can be taken as a representative of the rest of the lavage fluid, or whether the first clear sample of gut lavage effluent is just a bolus of material to study gut immunity. In the latter case, careful interpretation of the significance of the quantitative measurements of immunoglobulins and other components of WGL fluid would be required.

Aims of the study

1. To obtain at least 5 or 6 sequential clear specimens from every patient.

2. To examine the time at which these clear specimens can be obtained.

3. Whether or not sufficient volumes of clear lavage effluent are obtainable to be used for a variety of investigations.

4. To study various indices such as immunoglobulin contents, antigen-specific antibodies titres, alpha-1-antitrypsin, albumin etc. in the serial lavage specimens.

5. Finally and most importantly, to examine whether the first clear sample is representative of the rest of the lavage effluent or not i.e. whether gut lavage effluent is a perfusate or a bolus of material from the gut.

Patients studied

WGL was carried out in eleven patients (7 females and 4 males) were recruited from the out-patient clinic and from wards of the gastrointestinal unit (GIU). The age of patients ranged between 22 and 82 years (median age 61 years). Further details of patients involved in this study are described in table 4.I.

patient	age	sex	out-patient/ in-patient	diagnosis	remarks
1	28	M	out-patient	ulcerative colitis	
2	22	M	out-patient	ulcerative colitis	
3	61	M	out-patient	Crohn's disease	ileostomy
4	76	F	in-patient	colonic polyp	
5	82	F	in-patient	Crohn's disease	constipation
6	36	F	out-patient	Crohn's disease	ileostomy
7	64	F	in-patient	diarrhoea for inv.	
8	73	M	out-patient	ca rectum	constipation
9	61	F	in-patient	Crohn's disease	
10	23	F	in-patient	ulcerative colitis	
11	34	F	in-patient	Crohn's disease	

Table 4.I. Patients involved in the serial lavage study.

Approach

Patients attending the out-patient clinics of the GIU for a variety of GI diseases as well as patients in ward D1 and D2 who were admitted to the GIU either for treatment or for preparation for lower endoscopy were recruited after explaining the aims and the nature of the study. Written consents were obtained from all participants.

Methodology

1. After overnight fasting, patients started to drink the lavage solution at about 08.30 next morning. The nature of the lavage solution was described in the materials and methods chapter (chapter 3). Drinking of the lavage solution was at a regular rate of 200 mls (one glass) every 10-15 minutes over a period of 2-3 hours. The patients recorded the time when each glass was completed. A specimen of the lavage solution were taken into two universals to be used in the PEG assay later.

2. Within one to two hours the bowel of the subjects started to move. Solid and semi-liquid stools were discarded.

3. When the subjects passed clear fluid stools per rectum, the clear specimens were filtered and processed as described in chapter 3. Two unprocessed samples were also taken. Processed and unprocessed samples were put in a small box containing ice to reduce the activity of the proteolytic enzymes in the specimens till all 5 to 6 clear specimens were obtained then they were put at -70 °C as described in chapter 3.

4. Patients continued to drink the lavage solution till they passed at least 5 to 6 clear stool specimens.

5. The time at which every clear fluid stool specimen obtained was recorded and the volume of each clear specimen was measured.

Assays performed

The following assays were performed in 5 to 6 sequential lavage specimens obtained from the subjects. The methodology of these assays were described in chapter 3.

1. Standard ELISA was carried out for the quantitative measurements of TIgA, TIgM and TIgG. A modified ELISA was performed for the

quantitative measurements of anti-*Salmonella typhi* (LPS) antibody titres.

2. PEG assay.

3. Total proteins.

4. Albumin contents.

5. Alpha-1-antitrypsin.

6. Proteases contents in the processed and unprocessed samples.

Since the data obtained in this study were found to be non-parametrically distributed and paired, the Wilcoxon signed-rank test was used for the statistical analysis of the results.

Results

Three patients were excluded from the final analysis. These are patients number 4, 5 and 10. Patient number 4 was excluded because she only passed four small volume clear lavage specimens and failed to pass further

specimens. At least 2 lavage specimens obtained from patient number 5, an 82-year old lady, were found to be contaminated by urine. Patient number 10, a 23-year old lady with ulcerative colitis, had excessive bleeding per rectum during the experiment and the lavage specimens obtained from this lady were found to contain large amounts of haemoglobin. Graphical representation of total IgA, IgM and IgG in serial lavage samples of these three patients are shown in Figs. 4.XIII, 4.XIV and XV.

The first five sequential lavage specimens from each of the eight participants who continued the experiment successfully were used in the study.

Output of whole gut lavage

Generally, WGL technique was well-tolerated by all participants in this study. Once the patients started to pass clear lavage fluid per rectum, up to 5 to 6 good volumes of sequential clear fluid specimens could be obtained from every subject. The intake and output of lavage fluids obtained from 8 patients are shown in table 4.II and represented graphically in Figs. 4.I to 4.VIII.

Levels of various indices in five sequential lavage samples

1. **TIgA concentrations.** The levels of TIgA in five sequential lavage specimens obtained from every individual out of 8 patients showed no significant differences between samples number 1 and numbers 2, 3, 4, or 5 ($p=1.000$, 1.000, 0.529, and 0.834 for the differences between sample number 1 and numbers 2, 3, 4, and 5 respectively). Fig. 4.IX.

2. **Anti-*Salmonella typhi* (LPS) IgA antibody titres.** No significant differences were found between samples number 1 and numbers 2, 3, 4, or 5 ($p=0.624$, 0.441, 0.726, and 0.441 for the differences between samples number 1 and numbers 2, 3, 4, and 5 respectively). Fig. 4.X.

3. **TIgM concentrations.** No significant differences were found between samples number 1 and numbers 2, 3, 4, or 5 ($p=0.161$, 0.272, 0.076, and 0.080 for the differences between samples number 1 and numbers 2, 3, 4, and 5 respectively). Fig. 4.XI.

4. **TIgG concentrations.** No significant differences were found between samples number 1 and numbers 2 or 3 ($p=0.529$ and 0.363 for the differences between samples number 1 and numbers 2 and 3 respectively). However, the differences between samples number 1 and numbers 4 or 5 were found to be significant ($p=$ 0.025 and 0.021 for

the differences between samples number 1 and numbers 4 and 5 respectively). Fig. 4.XII.

5. **Albumin concentrations.** No significant differences were found between samples number 1 and numbers 2, 3, 4, or 5 ($p=0.933$, 0.612, 0.402, 0.612 for the differences between samples number 1 and numbers 2, 3, 4, and 5 respectively).

6. **Alpha-1-antitrypsin concentrations.** No significant differences were found between samples number 1 and numbers 2, 3, 4, or 5 ($p=1.000$, 1.000, 0.529, and 0.447 for the differences between samples number 1 and numbers 2, 3, 4, and 5 respectively).

7. **Proteases inhibition.** No significant differences were found in the percentage of inhibition of proteases activity between samples number 1 and numbers 2, 3, 4, or 5 ($p=1.000$, 0.201, 1.000, and 0.138 for the differences between samples number 1 and numbers 2, 3, 4, and 5 respectively).

8. **Total protein concentrations.** No significant differences were found between samples number 1 and numbers 2, 3, or 4 ($p=0.363$, 0.834, and 0.080 for the differences between samples number 1 and numbers 2, 3, and 4 respectively). However, the difference between samples

number 1 and number 5 was found to be significant ($p=0.022$) (table 4.III).

Conclusion

WGL has been shown to be a tolerable, non-invasive, and safe method that provides direct access to the intestinal secretions and a reliable method to investigate the gut immunity. Sufficient volumes of the lavage effluent were obtainable to allow various investigations of on the lavage output This experiment has shown that there are no significant differences between the concentrations of the various indices studied in the first clear sample passed per rectum in the WGL technique and the four succeeding sequential clear samples. Therefore, I concluded that the first clear sample obtained from WGL effluent is representative of the rest of the lavage effluent and, therefore, WGL fluid is a perfusate.

patient	intake			output (clear stools)		
	total (litres)	started (time)	completed	sample no.	sample time	sample volume(litres)
1	3.5	8.30	11.45	1	11.15	0.480
				2	12.00	0.380
				3	12.30	0.430
				4	12.55	0.450
				5	13.25	0.300
2	3.6	8.15	11.15	1	10.30	0.300
				2	11.10	0.220
				3	11.30	0.260
				4	12.05	0.900
				5	12.55	0.780
3	4.0	8.30	12.00	1	10.15	0.400
				2	10.55	0.410
				3	11.05	0.600
				4	11.30	0.300
				5	12.00	0.400
				6	12.30	0.300
6	1.75	8.35	9.55	1	9.15	0.075
				2	9.25	0.290
				3	9.35	0.155
				4	9.45	0.135
				5	9.50	0.215
				6	10.01	0.140
7	4.0	8.15	10.00	1	10.00	0.200
				2	10.15	0.200
				3	10.30	0.240
				4	10.40	0.140
				5	10.50	0.200
				6	10.56	0.120
8	5.5	8.00	14.30	1	14.15	0.150
				2	14.40	0.200
				3	15.00	0.400
				4	15.15	0.250
				5	15.35	0.200
				6	15.50	0.175

Table 4.II. Intake and output of lavage fluids of 8 patients involved in the serial lavage study (continued below). Patients 4, 5 and 10 were excluded (see text).

9	4.5	9.45	15.00	1	15.00	0.280
				2	15.15	0.160
				3	15.30	0.180
				4	15.40	0.200
				5	15.50	0.150
				6	16.00	0.060
11	3	9.35	13.15	1	11.45	0.600
				2	12.15	0.400
				3	12.30	0.100
				4	12.50	0.140
				5	13.00	0.200
				6	13.20	0.200

Table 4.II. (continued) Intake and output of lavage fluids of 8 patients involved in the serial lavage study.

patient	sample 1	sample 2	sample 3	sample 4	sample 5
1	1850	1620	1500	1200	1400
2	1700	1940	1400	1000	1000
3	500	600	400	420	420
4	540	553	560	560	460
5	330	480	420	370	330
6	450	500	710	440	410
7	830	880	1190	680	670
8	560	470	460	420	440

Table 4.III. Values of total proteins in the serial lavage study (μg/ml). No significant differences were found between samples number 1 and numbers 2, 3 or 4 (p values were 0.363, 0.834 and 0.080 respectively), but the difference between samples number 1 and number 5 was found to be significant (p value was 0.022).

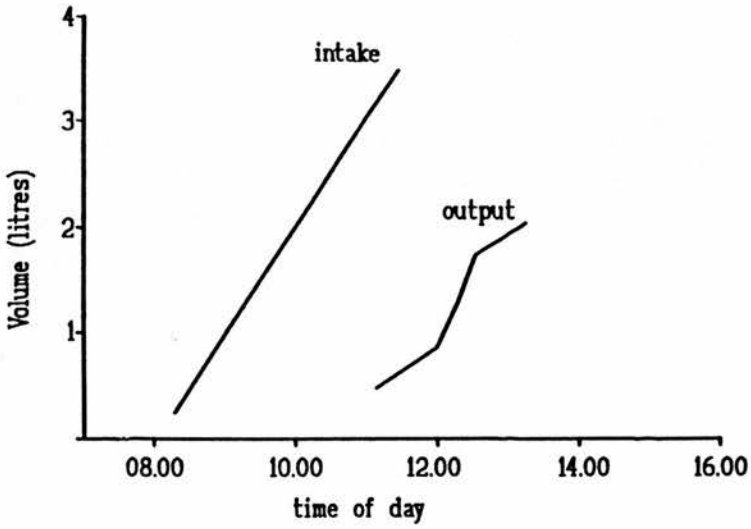


Fig. 4.I. Intake and output of lavage fluid of patient 1 involved in the serial lavage study.

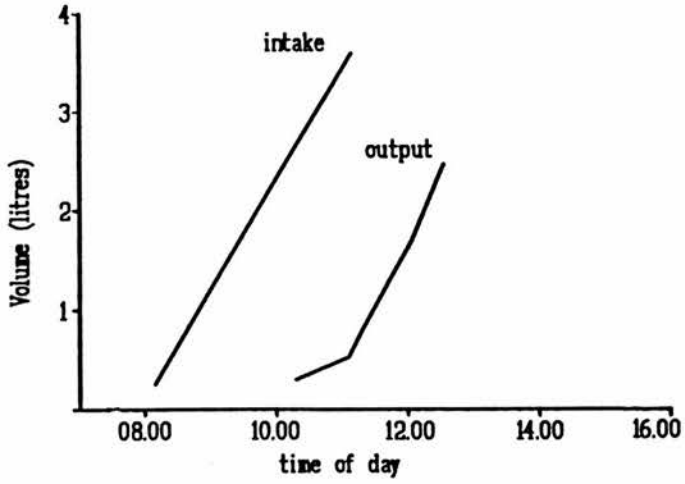


Fig. 4.II. Intake and output of lavage fluid of patient 2 involved in the serial lavage study.

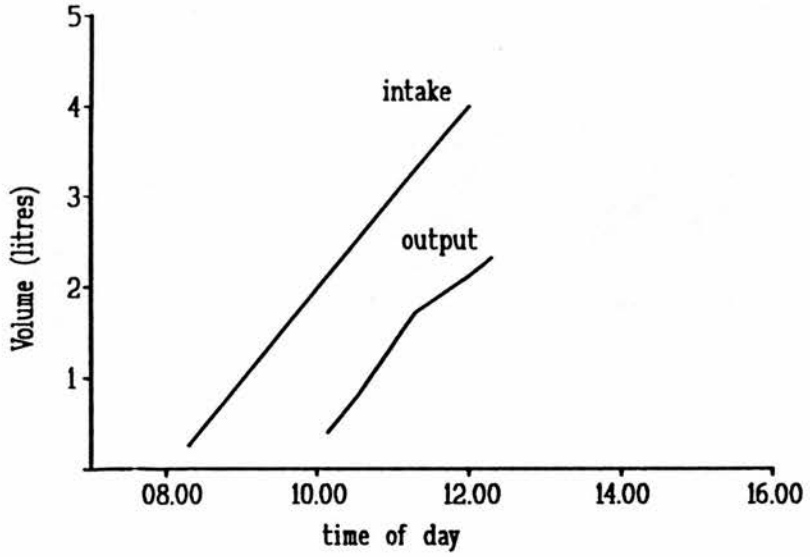


Fig. 4.III. Intake and output of lavage fluid of patient 3 involved in the serial lavage study. This patient had an ileostomy.

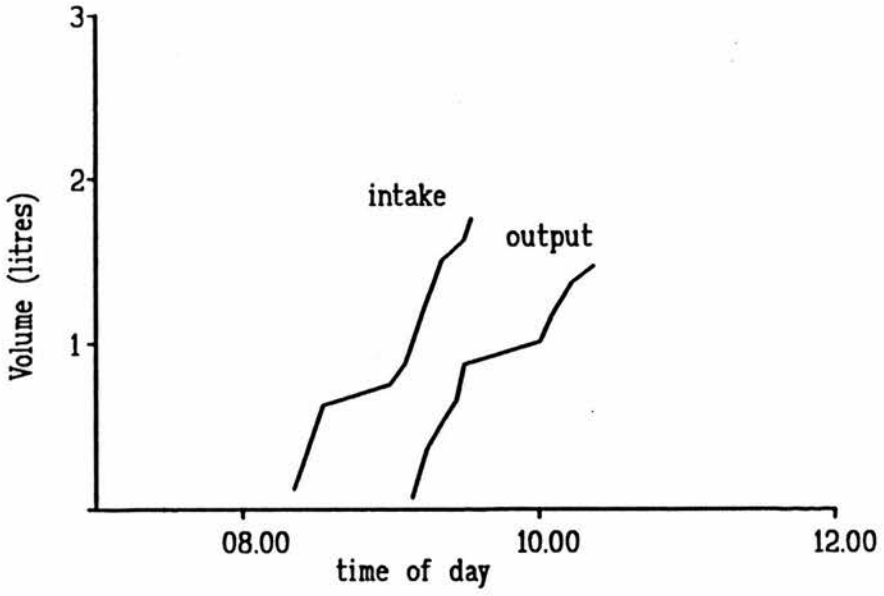


Fig. 4.IV. Intake and output of lavage fluid of patient 4 involved in the serial lavage study. This patient had an ileostomy.

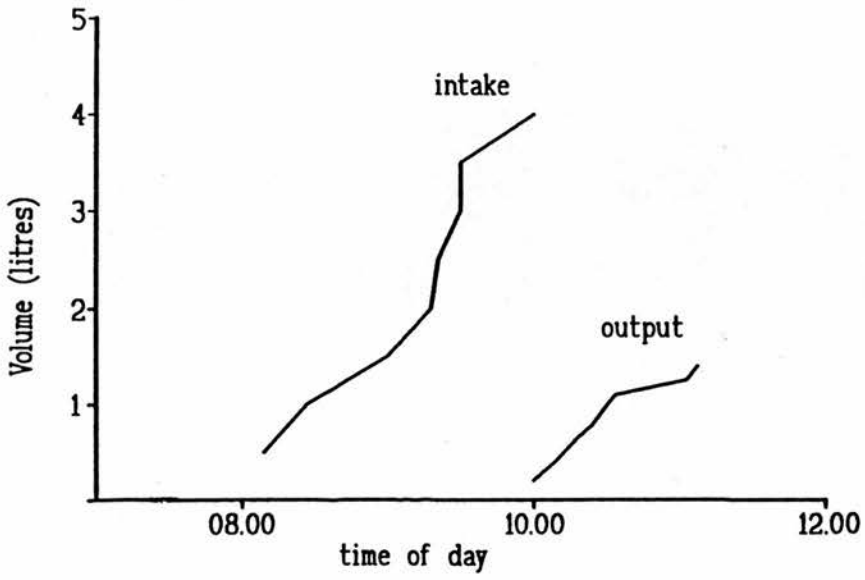


Fig. 4.V. Intake and output of lavage fluid of patient 5 involved in the serial lavage study.

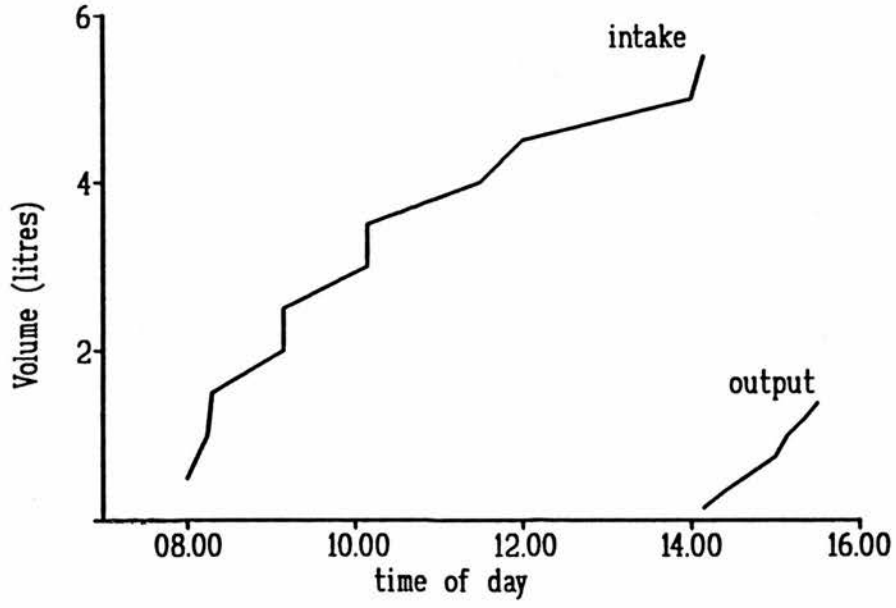


Fig. 4.VI. Intake and output of lavage fluid of patient 6 involved in the serial lavage study. This patient had intractable constipation.

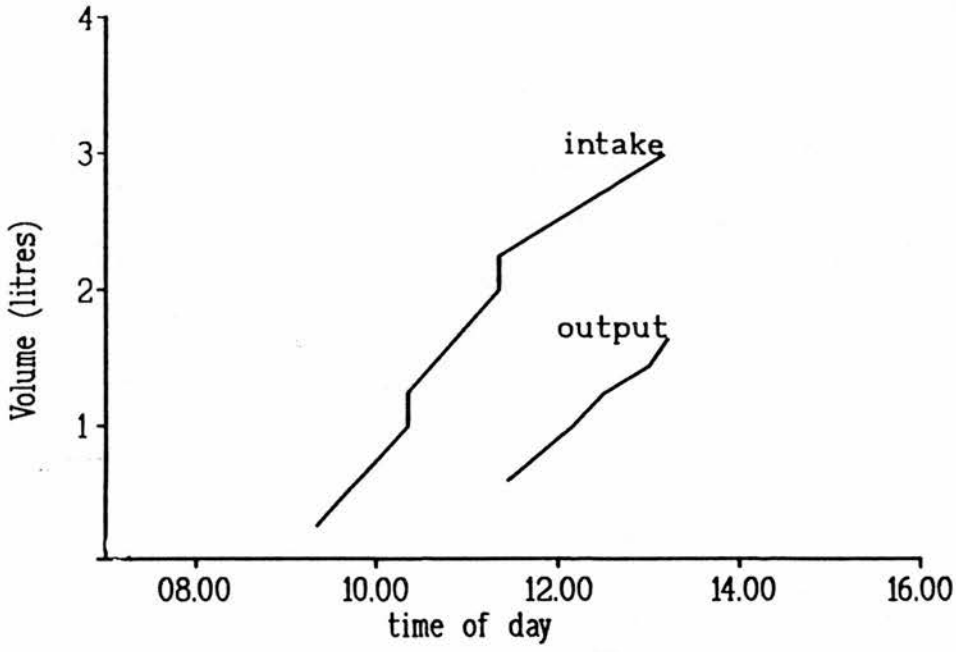


Fig. 4.VII. Intake and output of lavage fluid of patient 7 involved in the serial lavage study.

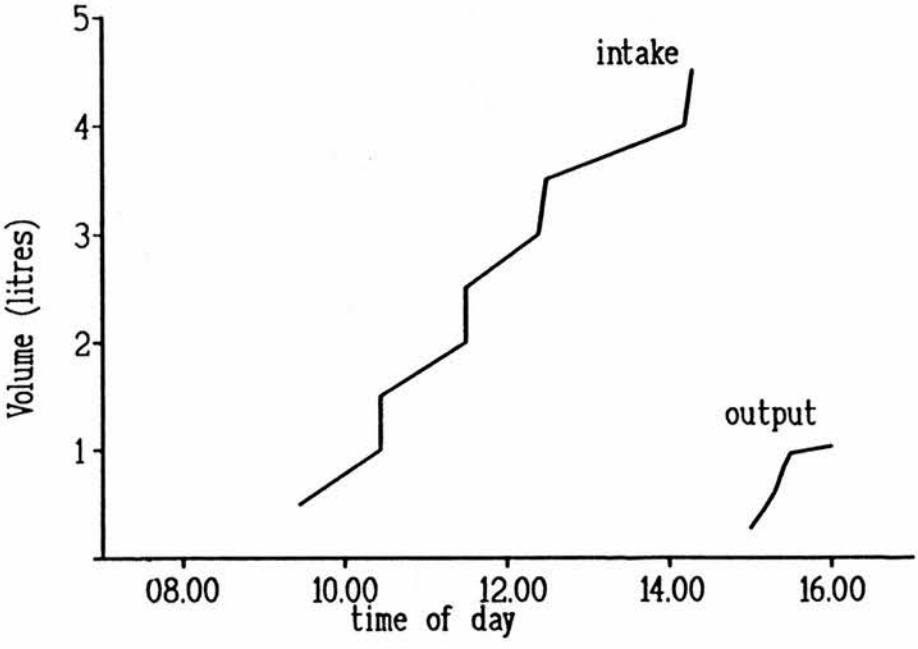


Fig. 4.VIII. Intake and output of lavage fluid of patient 8 involved in the serial lavage study.

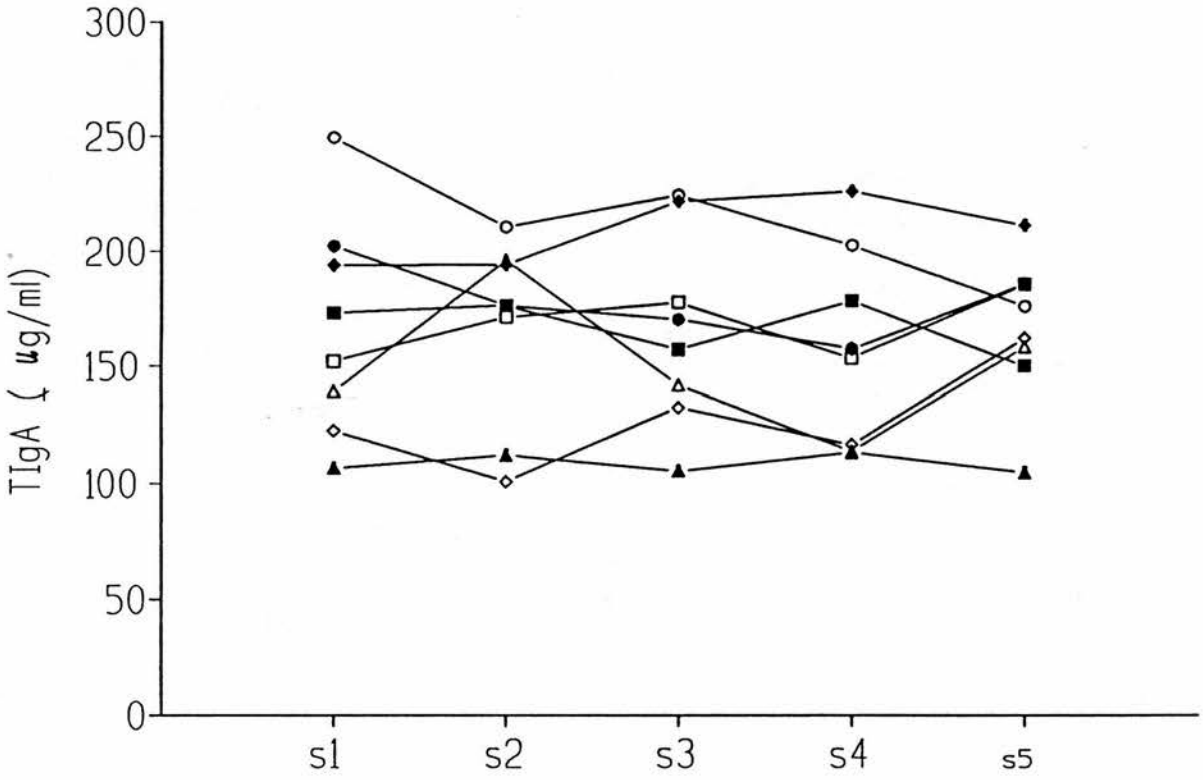


Fig. 4.IX. Concentrations of total IgA (TIgA) in 5 sequential lavage samples of 8 patients involved in the serial lavage study. S=sample

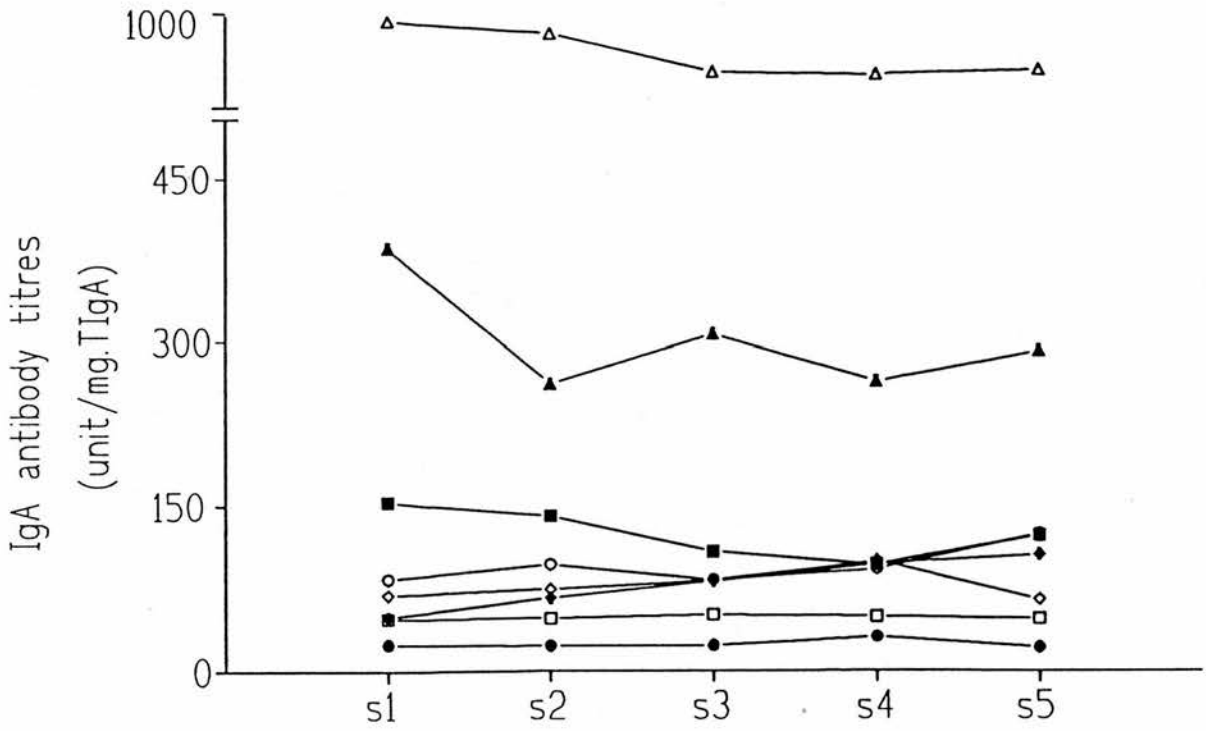


Fig. 4.X. Concentrations of anti-*Salmonella typhi* LPS IgA antibodies in 5 sequential lavage samples of 8 patients involved in the serial lavage study. S=sample.

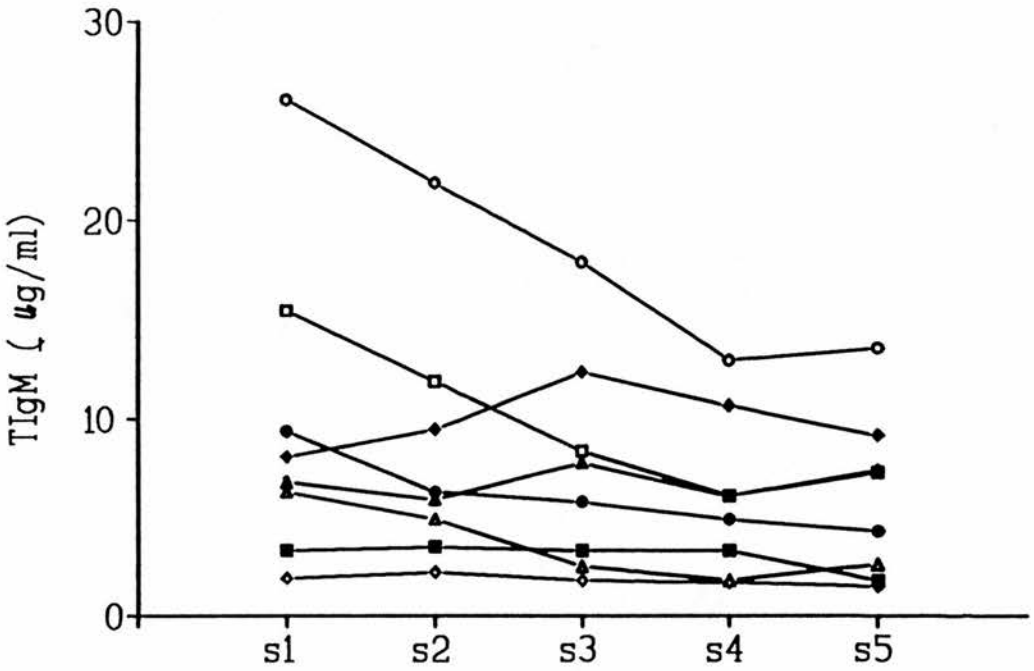


Fig. 4.XI. Concentrations of total IgM (TIgM) in 5 sequential lavage samples of 8 patients involved in the serial lavage study. S=sample.

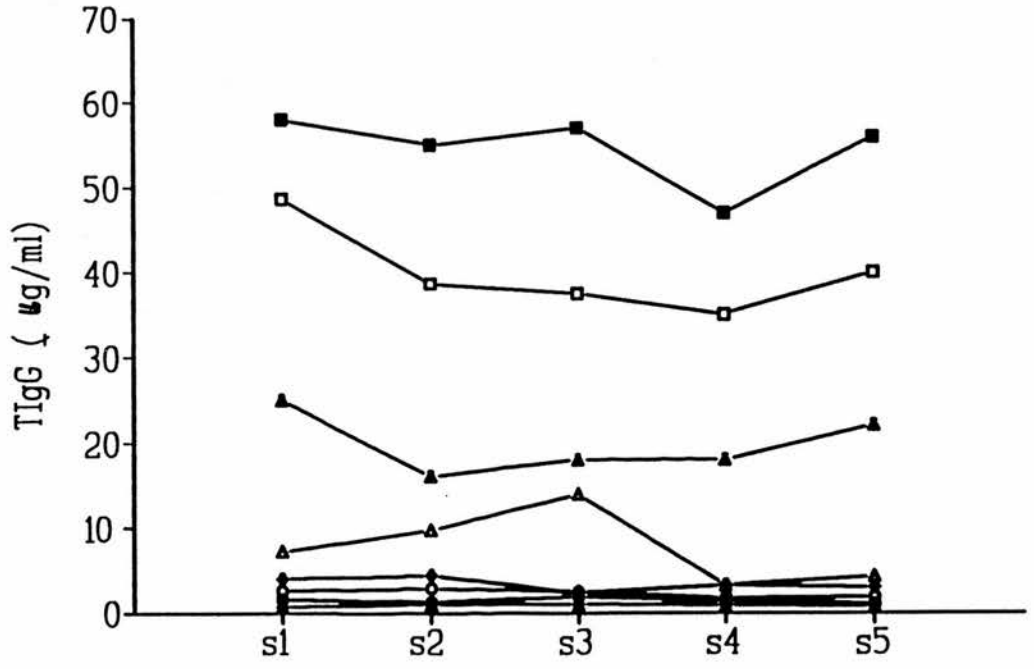


Fig. 4.XII. Concentrations of total IgG (TIgG) in 5 sequential lavage samples of 8 patients involved in the serial lavage study. S=sample.

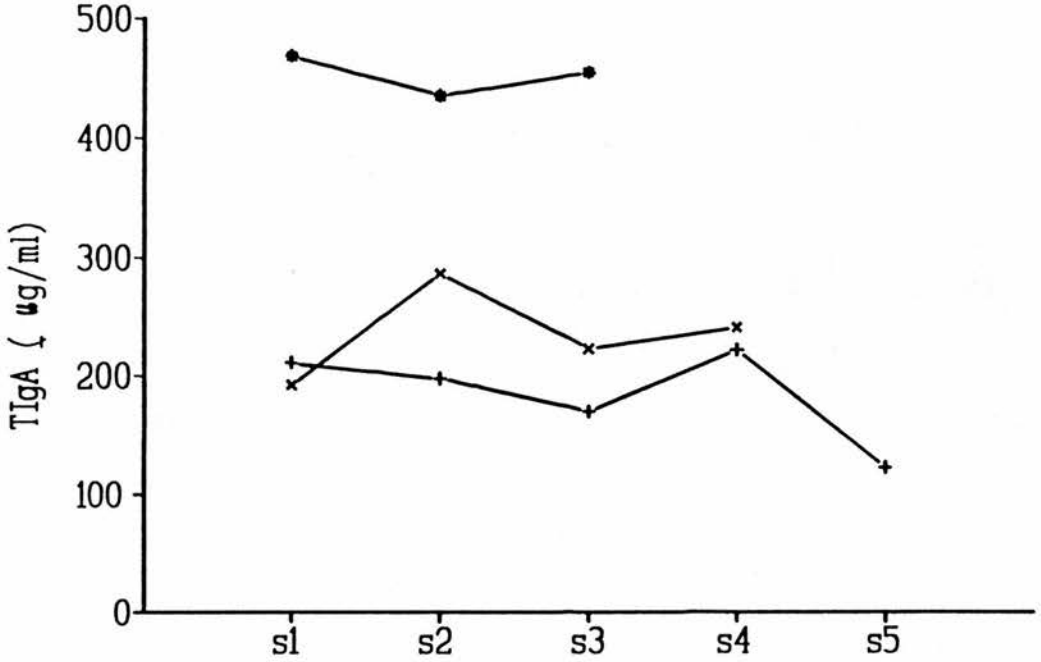


Fig. 4.XIII. Concentrations of TIgA in sequential lavage samples of 3 patients excluded from the final analysis of the results in the serial lavage study.

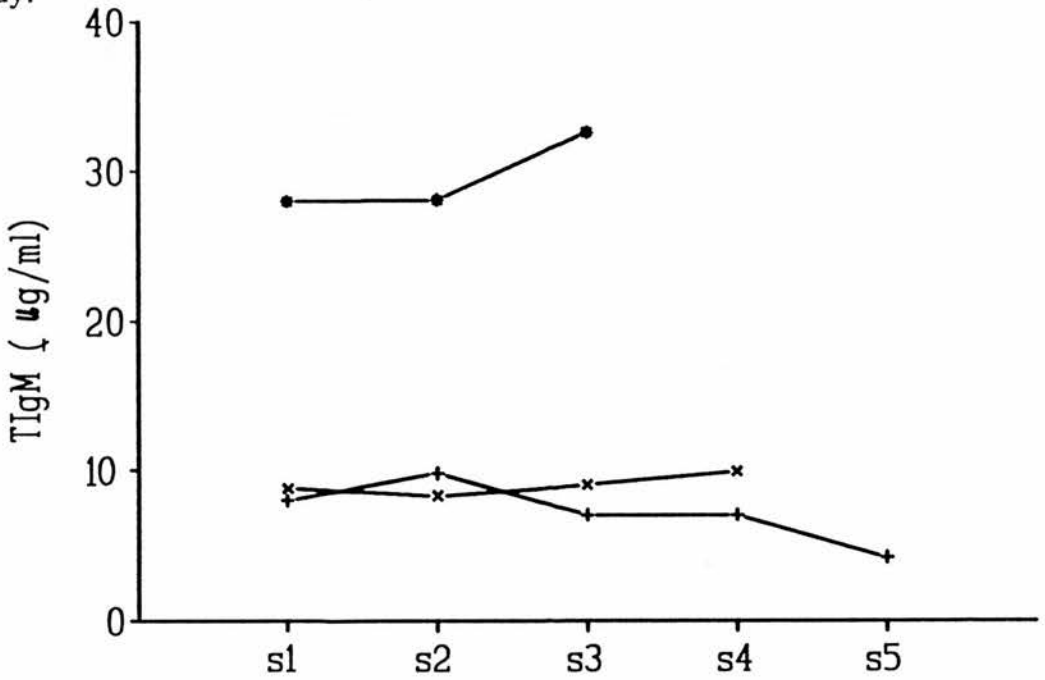


Fig. 4.XIV. Concentrations of TIgM in sequential lavage samples of 3 patients excluded from the final analysis of the results in the serial lavage study.

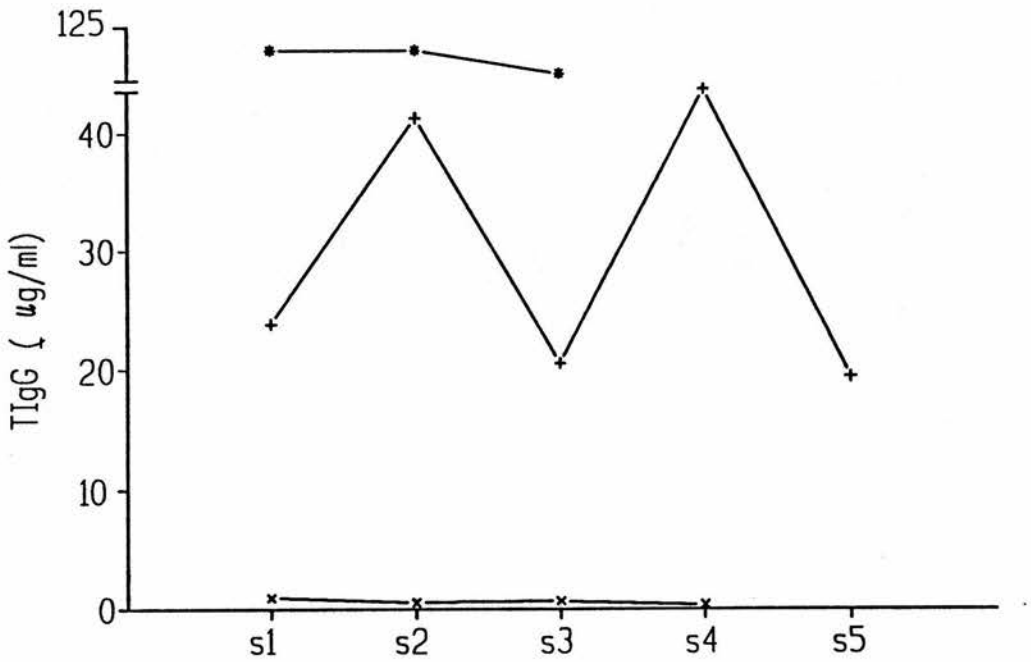


Fig. 4.XV. Concentrations of TIgG in sequential lavage samples of 3 patients excluded from the final analysis of the results in the serial lavage study.

Chapter V

INTESTINAL IgA, IgM AND IgG IN INFLAMMATORY BOWEL DISEASE AS COMPARED TO HEALTHY VOLUNTEERS

Background

Inflammatory bowel disease (IBD) consists of two major illnesses, ulcerative colitis (UC) and Crohn's disease (CD), and is characterised by chronic inflammation of the intestine. Both diseases, UC and CD, have been described as chronic inflammatory diseases because of the prolonged clinical course and due to the fact that inflammatory infiltrate contains lymphocytes and macrophages, a histological picture of chronic inflammation. During acute exacerbation (active stage of IBD), the inflamed mucosa contains large numbers of acute inflammatory cells such as neutrophils and monocytes which then pass to the intestinal lumen (Schreiber et al, 1992).

The aetiological agent(s) for UC and CD is/are, as yet, unknown. A large number of microbial and dietary agents have been suggested as candidates for the aetiology of UC and CD. However, none of those agents has been proved to be the causative agent of either UC or CD. UC is characterised by mucosal ulcers and the infiltration of the mucosa and submucosa with neutrophils, macrophages and lymphocytes while the histopathological lesion of CD is characterised by granulomas which extend through all layers of the bowel wall (MacDermott and Stenson, 1990).

Immunopathology of IBD

Peripheral blood lymphocyte function. The total number of the peripheral blood lymphocytes (PBLs) of patients with IBD has been found not to significantly differ from normal controls (Selby and Jewell, 1983). However, the functional state and the degree of activation of PBLs may be changed. Using the T9 antigen, which is the transferrin receptor and is expressed during early lymphocyte activation, Raedler and colleagues (1985) have demonstrated that 24% of PBLs of patients with active CD expressed early activation markers and only 10% of PBLs expressed the T9 antigen in inactive CD. In another study, Pallone et al (1987) demonstrated that, in CD, T9 antigen was expressed in increased proportions on both peripheral blood mononuclear cells (MNCs) and isolated intestinal lamina propria MNCs. Mueller and co-workers (1990) showed that levels of soluble interleukin-2 receptors (IL-2R) in sera of patients with CD were increased. Soluble IL-2R appear on the surface of T cells and macrophages during cellular activation (Schreiber et al, 1992). However, activation of PBLs was found to be not specific for IBD and was observed also in a variety of autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and Behcet's disease, but not in bacterial or viral colitis (Raedler et al, 1985).

Intestinal lymphocyte function. IBD is characterised by a mixed cellular infiltrate consisting mainly of B cells and T cells. B cells are found in the mucosa close to IBD ulcers while T cells are found around granulomas and in the submucosa of CD

lesions (Schreiber et al, 1992). In addition, neutrophils, eosinophils, mast cells and macrophages are also encountered in IBD lesions. Immunohistological studies have shown that IBD is associated with increased expression, on intestinal B cells and T cells, of lymphocyte activation antigens such as 4F2, transferrin receptors, IL-2 receptors and HLA-DR (Pallone et al, 1987; Allison et al, 1984; MacDonald et al, 1990). Allison et al (1984) demonstrated also the appearance of CD7 as a marker for the increased T cell activation in IBD. Furthermore, $CD4^+$ and $CD8^+$ T lymphocyte subpopulations were found to be increased in both active UC and CD (Schreiber et al, 1991). Generally, the above studies have shown that IBD is associated with increased activity of B cells and T cells in the intestine as well as in the peripheral blood of patients with UC and CD.

Intestinal immunoglobulins synthesis and secretion in IBD

In the normal mucosa, IgA-producing B cells are the predominant type of immunoglobulin-producing cells, being 20 times as numerous as IgG-producing cells (Crabbe et al, 1970). They represent 70-90% of the total immunoglobulin-producing cells in the intestine (Brandtzaeg and Baklien, 1976). Intestinal MNCs isolated from normal mucosa have been shown to secrete large amounts of IgA spontaneously (MacDermott et al, 1983; MacDermott et al, 1986) and to have different functional characteristics from peripheral blood MNCs which have been

found to produce little IgA, IgG and IgM spontaneously (MacDermott et al, 1983; MacDermott et al, 1981; MacDermott, 1988).

In IBD, the mucosal immune system shows an altered immuno-regulation with a marked increase in IgG-containing cells. *In vitro* studies have shown that the total numbers of lymphocytes in intestinal specimens from IBD patients were four times greater than in normal mucosa with the major increase occurring in IgG-containing cells (Brandtzaeg et al, 1974; Baklien and Brandtzaeg, 1975; Rosedrans et al, 1980; Scott et al, 1983; Van Spreeuwel et al, 1985). Compared with normal control specimens, numbers of IgG-containing cells were found to be 30 times greater while numbers of IgA-containing cells were found to be 2 times greater and those of IgM-containing cells were 5 times greater than in normal control specimens (Brandtzaeg et al, 1974; Baklien and Brandtzaeg, 1975).

Studies on total IgG (TIgG) and IgG subclasses in the mucosa of IBD patients, showed that there was an increase in IgG1-containing cells in the mucosa of UC patients while the mucosa of patients with CD contained an increased number of IgG2-containing cells (Kett et al, 1987). Compared to intestinal MNCs of normal controls, MNCs of IBD patients exhibited a marked increase in spontaneous secretion of TIgG with increased proportions of IgG1 in MNCs from UC patients and IgG2 in MNCs from CD patients (Scott et al, 1986).

The differences in the IgG subclasses expression between the 2 diseases suggest that there may be different antigenic stimuli since IgG1 (and IgG3) are the dominant antibodies in the immune response to proteins and T cell-dependent antigens (Skakib and Stanworth, 1980; Heiner, 1984; Oxelius, 1984) whereas the production of IgG2 antibody is mainly stimulated by carbohydrate and bacterial antigens (Schreiber et al, 1992). This may account for the recurrent bacterial infections such as otitis media, pneumococcal respiratory infections, pericarditis and meningococcal infections reported to be associated with IgG2 and IgG4 deficiencies (Skakib and Stanworth, 1980; Heiner, 1984; Oxelius, 1984). Whether the increased production of IgG and IgG subclasses represents defined responses against specific antigens or merely up-regulation of non-specific responses to common luminal antigens remains not well-understood.

In contrast to IgG, there have been conflicting reports regarding IgA synthesis and secretion by the intestinal mucosa of IBD patients. MacDermott et al (1981; 1983) reported decreased spontaneous IgA secretion by the isolated MNCs of intestinal mucosa from IBD patients compared to normal controls. Furthermore, MNCs of intestinal mucosa from IBD patients were found to produce more IgA1 (up to 71-74% of the total IgA) than IgA2 while the MNCs of intestinal mucosa from normal controls produced more IgA2 (up to 61% of the total IgA) (MacDermott et al, 1986). Conversely, in one *in vitro* study, Badr El-Din et al (1988) reported a significant increase in the number of IgA-plasma cells and in the production of total IgA (TIgA) by colonic mucosa from patients with quiescent UC as compared

to normal controls, but no concomitant increase in the amount of secretory IgA (SIgA) was observed in the culture.

In a pilot study of the immunoglobulin concentrations in WGL fluid, O'Mahony et al (1990) reported that no significant differences were found between levels of TIgA in WGL fluid of patients with CD and the normal controls while levels of IgG and IgM were found to be significantly higher in the patients group compared to the normal control group. Similar results were reported by Mwantembe (1992) who found that, regardless of disease activity, there were no significant differences between levels of TIgA in WGL fluid of patients with IBD and normal controls.

However, the individuals used as a control group in the study of O'Mahony and colleagues (1990) were patients who underwent WGL as a preparation for barium enema or colonoscopy after presenting with one or more gastrointestinal problem(s) such as irritable bowel syndrome (IBS) or constipation. Although these individuals were reported to be "immunologically normal", this does not exclude the fact that they were not healthy and using them as a normal control may produce confusing results. The number of patients with CD involved in the study of O'Mahony et al was relatively small (10 patients). Furthermore, the technique of WGL was at its early stages and further improvements have been introduced into the methods of administering the lavage solution (supervised by special nurse) and processing of the lavage effluent. Results obtained by Mwantembe were widely scattered and the

control group used for the comparative study was similar to that used by O'Mahony.

Therefore, further research into the intestinal immune status of patients with IBD by investigating levels of intestinal immunoglobulins, particularly IgA, has become necessary. Furthermore, levels of intestinal immunoglobulins of patients with active and quiescent IBD should be compared with levels of immunoglobulins in the intestinal fluid of well characterised healthy individuals.

Aims of the study

In the light of the above studies reporting conflicting results on the intestinal immunoglobulins, particularly IgA, of patients with IBD, one aim of the study was to investigate the intestinal immune status of patients with active and inactive IBD by studying levels of immunoglobulins in their WGL fluid and comparing them with British adult healthy volunteers. The other aim of the study was to investigate levels of intestinal immunoglobulins in WGL fluid of healthy individuals since personal communication with Professor Svennerholm (University of Göteborg, Sweden) indicated that levels of intestinal TIgA in healthy Swedish individuals were much lower than those levels in the so called "normal patients" previously reported from this centre.

Therefore, for all the above reasons it was important to re-examine TIgA, TIgM and TIgG in healthy individuals. I did not intend to carry out an extensive analysis of IBD cases since such work is being carried out by other researchers in this centre who are investigating the immunoregulatory mechanisms in IBD.

Patients and methods

Specimens of WGL fluid obtained from a group of 22 British adult healthy volunteers (2 females and 20 males) with a median age of 27 years (ranged between 22 and 44) were used as a normal control. This group is described in more detail in chapter 6. WGL specimens obtained from patients who underwent WGL during the year 1992 were reviewed and lavage samples found to be from patients confirmed to have IBD (CD or UC) were selected. Most of these patients underwent WGL as a bowel preparation for barium enema, colonoscopy and/or bowel surgery. WGL specimens were stored at -70 ^{o}C after being collected and processed as described in chapter 3. A total of 59 specimens were found to be of IBD patients (32 females and 27 males) with a median age of 40 years, ranged between 12 and 79. IBD patients were divided into 40 patients (23 females and 17 males) with CD and 19 patients (9 females and 10 males) with UC. Patients with CD were further divided into subgroups of 16 patients (9 females and 7 males) with active CD and 24 patients (14 females and 10 males) with inactive CD. Likewise, patients with UC were further divided into subgroups of 9 patients (4

females and 5 males) with active UC and 10 patients (5 females and 5 males) with inactive UC.

Assessment of disease activity was based on the concentrations of TIgG in WGL fluid of IBD patients. Previous work in this centre (Choudari et al, 1993; Ferguson et al, 1994; Brydon et al, 1993; O'Mahony et al, 1991b) found that a level of > 10 μg/ml TIgG in WGL fluid was almost always consistent with clinical assessment of active IBD while WGL fluid obtained from patients with inactive IBD and normal individuals contained lower levels of TIgG.

Quantitative measurement of the concentrations of total IgA, IgM and IgG in WGL specimens obtained during the year 1992 and specimens of the healthy volunteers were carried out using ELISA. The technical details were as described in chapter 3.

Since all results obtained in this study were found to be non-parametrically distributed, the Mann-Whitney U-test was used for the statistical analysis of the data.

Results

The numerical data in sections 1-4 below are also shown in table 5.I.

1. **Concentrations of total IgA, IgM and IgG in WGL fluid of healthy volunteers**. The medians of the concentrations of total immunoglobulins in WGL fluid were 73.25 μg/ml with a range of 10.00-173.20, 2.2 μg/ml with a range of 0.1-9.5 and 1.0 μg/ml with a range of 1.0-2.0 for TIgA, TIgM and TIgG respectively.

2. **Concentrations of total IgA, IgM and IgG in WGL fluid of IBD patients as a whole**. When all 59 CD and UC patients were studied together as one group, the medians of the concentrations of total immunoglobulins in WGL fluid were 146.0 μg/ml with a range of 31.0-520.0, 5.1 μg/ml with a range of 1.1-80.9 and 7.1 μg/ml with a range of 0.4-280.3 for TIgA, TIgM and TIgG respectively.

3. **Concentrations of total IgA, IgM and IgG in WGL fluid of patients with Crohn's disease**. As one group, patients with CD had the following medians (and ranges): 137.0 μg/ml (49.0-520.0), 6.0 μg/ml (1.1-80.9) and 7.05 μg/ml (0.5-280.0) for TIgA, TIgM and TIgG respectively. When patients with CD were further divided into subgroups of active or inactive CD patients, they were found to have the following medians (and ranges): (a) active CD patients had medians of 154.0 μg/ml (65.0-413.0), 6.95 μg/ml (2.30-28.50) and 30.3 μg/ml (11.4-280.3) for TIgA, TIgM and TIgG respectively; (b) inactive CD

patients had medians of 105.0 μg/ml (49.0-520.0), 4.5 μg/ml (1.1-80.9) and 3.35 μg/ml (0.50-9.00) for TIgA, TIgM and TIgG respectively.

4. **Concentrations of total IgA, IgM and IgG in WGL fluid of patients with ulcerative colitis**. As one group, patients with UC were found to have the following medians (and ranges): 236.0 μg/ml (31.0-453.0), 4.60 μg/ml (2.30-43.70) and 7.30 μg/ml (0.40-236.40) for TIgA, TIgM and TIgG respectively. When patients with UC were further divided into subgroups of active or inactive UC patients, they were found to have the following medians (and ranges): (a) active UC patients had medians of 294.0 μg/ml (68.0-453.0), 8.90 μg/ml (2.50-43.70) and 52.5 μg/ml (17.0-236.4) for TIgA, TIgM and TIgG respectively; (b) inactive UC patients had medians of 92.0 μg/ml (31.0-365.0), 3.90 μg/ml (2.30-16.50) and 1.90 μg/ml (0.40-7.30) for TIgA, TIgM and TIgG respectively.

5. **Comparison between levels of total IgA in WGL fluid of healthy volunteers and patients with Crohn's disease**. When levels of TIgA in WGL fluid of healthy volunteers were compared with levels of TIgA in WGL fluid of patients with CD regardless of disease activity, patients with CD were found to have significantly higher levels of TIgA than had healthy volunteers ($p=0.0003$) (table 5.I and Fig. 5.I). When patients with CD were divided into patients with active or inactive CD, patients with either active or inactive CD were found to

have significantly higher levels of TIgA than had healthy volunteers (p values were 0.0003 and 0.006 for active and inactive CD versus healthy volunteers respectively) (table 5.I and Fig. 5.IV).

6. **Comparison between levels of total IgA in WGL fluid of healthy volunteers and patients with ulcerative colitis.** When levels of TIgA in WGL fluid of healthy volunteers were compared with levels of TIgA in WGL fluid of patients with UC regardless of disease activity, patients with UC were found to have significantly higher levels of TIgA than had healthy volunteers ($p=0.003$) (table 5.I and Fig. 5.I). When patients with UC were further divided into patients with active or inactive UC, levels of TIgA were found to be significantly higher in WGL fluid of patients with active UC than in healthy volunteers ($p=0.0003$) but no significant differences were found between levels of TIgA in WGL fluid of patients with inactive UC and those of healthy volunteers ($p=0.208$) (table 5.I and Fig. 5.IV).

7. **Comparison between levels of total IgM in WGL fluid of healthy volunteers and patients with Crohn's disease.** As one group, patients with CD were found to have significantly higher levels of TIgM than did healthy volunteers ($p=0.0001$) (table 5.I and Fig. 5.II). Likewise, when patients with CD were divided into patients with active or inactive CD, levels of TIgM in patients with CD, regardless of disease activity, were found to be significantly higher than those of healthy volunteers (p values were 0.0001 and 0.001 for

active and inactive CD versus healthy volunteers respectively) (table 5.I and Fig. 5.V).

8. **Comparison between levels of total IgM in WGL fluid of healthy volunteers and patients with ulcerative colitis**. As one group, patients with UC were found to have significantly higher levels of TIgM than had healthy volunteers (p=0.0002) (table 5.I and Fig. 5.II). When patients were divided into patients with active or inactive UC, patients with UC, regardless of disease activity, were found to have significantly higher levels of TIgM than had healthy volunteers (p values were 0.0006 and 0.010 for active and inactive UC versus healthy volunteers respectively) (table 5.I and Fig. 5.V).

9. **Comparison between levels of total IgG in WGL fluid of healthy volunteers and patients with Crohn's disease**. As one group, patients with CD were found to have significantly higher levels of TIgG than had healthy volunteers (p=0.0001) (table 5.I and Fig 5.III). Regardless of disease activity, levels of TIgG in WGL fluid of patients with CD were found to be significantly higher than in healthy volunteers (p values were 0.0001 and 0.0003 for active and inactive CD versus healthy volunteers respectively) (table 5.I and Fig 5.VI).

10. **Comparison between levels of total IgG in WGL fluid of healthy volunteers and patients with ulcerative colitis**. As one group, patients with UC were found to have higher levels of TIgG than had healthy volunteers (p=0.0001)

(table 5.I and Fig. 5.III). Likewise, regardless of disease activity, patients with UC were found to have higher levels of TIgG than had healthy volunteers (p values were 0.0001 and 0.034 for active and inactive UC versus healthy volunteers respectively) (table 5.I and Fig. 5.VI).

11. **Comparison between levels of total IgA and IgM in WGL fluid of patients with active and inactive Crohn's disease.** When levels of TIgA in WGL fluid of patients with active CD were compared with those of patients with inactive CD, no significant differences were found between the 2 subgroups ($p=0.1633$). Likewise, no significant differences were found between levels of TIgM in WGL fluid of patients with active and inactive CD ($p=0.1633$) (table 5.II).

12. **Comparison between levels of total IgA and IgM in WGL fluid of patients with active and inactive ulcerative colitis.** No significant differences were found between levels of TIgA in WGL fluid of patients with active and inactive UC ($p=0.094$). Likewise, no significant differences were found between levels of TIgM in WGL fluid of patients with active and inactive UC ($p=0.094$) (table 5.II).

Conclusion

Immunoglobulin A (IgA) was found to be the predominant immunoglobulin in WGL fluid of both healthy volunteers and IBD patients. While WGL fluid of the healthy volunteers contained only traces of TIgM and TIgG, WGL fluid of patients with IBD contained large amounts of TIgM and, in particular, TIgG. Compared to healthy volunteers, and regardless disease activity, WGL fluid of patients with CD and UC contained significantly higher levels of TIgA, TIgM and TIgG. Patients with active CD or UC had significantly higher levels of TIgA, TIgM and TIgG in WGL fluid than had healthy volunteers. While patients with inactive CD had significantly higher levels of TIgA, TIgM and TIgG than did healthy volunteers, patients with inactive UC had significantly higher levels of TIgM and IgG than did healthy volunteers but no significant differences were found between levels of TIgA in WGL fluid of healthy volunteers and patients with inactive UC.

Within the subgroups of CD and UC patients, no significant differences were found between levels of TIgA or IgM in WGL fluid of patients with active CD and patients with inactive CD. Likewise, no significant differences were found between levels of TIgA or TIgM in WGL fluid of patients with active UC and patients with inactive UC.

The above findings indicated that IBD is associated with major alteration in levels of intestinal immunoglobulins, particularly TIgG. The intestinal secretions of

patients with IBD exhibited a marked polyclonal up-regulation of IgA, IgM and IgG. Whether the increase in polyclonal immunoglobulins in IBD patients was due to leakage from the systemic circulation or due to increase in local production is, as yet, unknown. Since levels of TIgA and TIgM in WGL fluid of patients with active IBD overlapped with those of inactive IBD, these levels can not be used as indices for disease activity in IBD.

Accurate and reliable analysis of intestinal immunoglobulins in IBD patients may provide an important clue(s) into the pathophysiology and immunopathology of IBD.

immunoglobulins and *p* (versus healthy volunteers)	healthy volunteers n=22	IBD (all) n=59	CD (all) n=40	UC (all) n=19	active CD n=16	inactive CD n=24	active UC n=9	inactive UC n=10
IgA median	73.25 μg/ml	146.0 μg/ml	137.0 μg/ml	236.0 μg/ml	154.0 μg/ml	105.0 μg/ml	294.0 μg/ml	92.0 μg/ml
range	10.00-173.20	31.0-520.0	49.0-520.0	31.0-453.0	65.0-413.0	49.0-520.0	68.0-453.0	31.0-365.0
p	not applicable	0.0001	0.0003	0.003	0.0003	0.006	0.0003	0.208
IgM median	2.20 μg/ml	5.10 μg/ml	6.0 μg/ml	4.60 μg/ml	6.95 μg/ml	4.5 μg/ml	8.90 μg/ml	3.90 μg/ml
range	0.10-9.50	1.10-80.90	1.1-80.9	2.30-43.70	2.30-28.50	1.1-80.9	2.50-43.70	2.30-16.50
p	not applicable	0.0001	0.0001	0.0002	0.0001	0.001	0.0006	0.010
IgG median	1.0 μg/ml	7.10 μg/ml	7.05 μg/ml	7.30 μg/ml	30.3 μg/ml	3.35 μg/ml	52.5 μg/ml	1.90 μg/ml
range	1.0-2.0	0.40-280.30	0.5-280.3	0.40-236.40	11.4-280.3	0.50-9.00	17.0-236.4	0.40-7.30
p	not applicable	0.0001	0.0001	0.0001	0.0001	0.0003	0.0001	0.034

Table 5.I. A summary of the results of measurements of total immunoglobulins in WGL fluid of British healthy volunteers and IBD patients.

group	IgA concentration (p versus inactive disease)	IgM concentration (P versus inactive disease)
active CD	0.1633	0.1633
active UC	0.0942	0.0942

Table 5.II. Comparison between levels of TIgA, TIgM and TIgG in WGL fluid of patients with active and inactive IBD (p values of the medians).

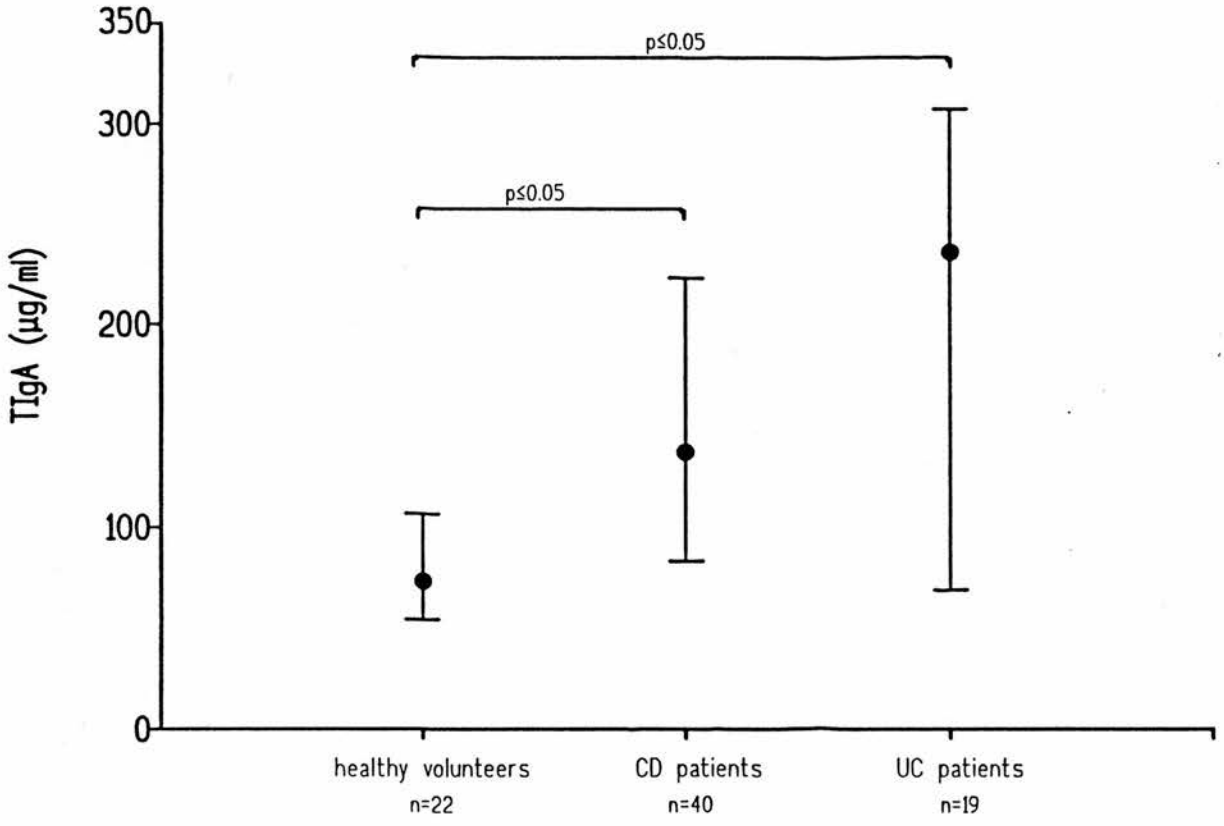


Fig. 5.I. Concentrations of TIgA in WGL fluid of healthy volunteers compared to patients with Crohn's disease (CD) and ulcerative colitis (UC). •=Median. Bars show the upper and lower quartiles of the ranges.

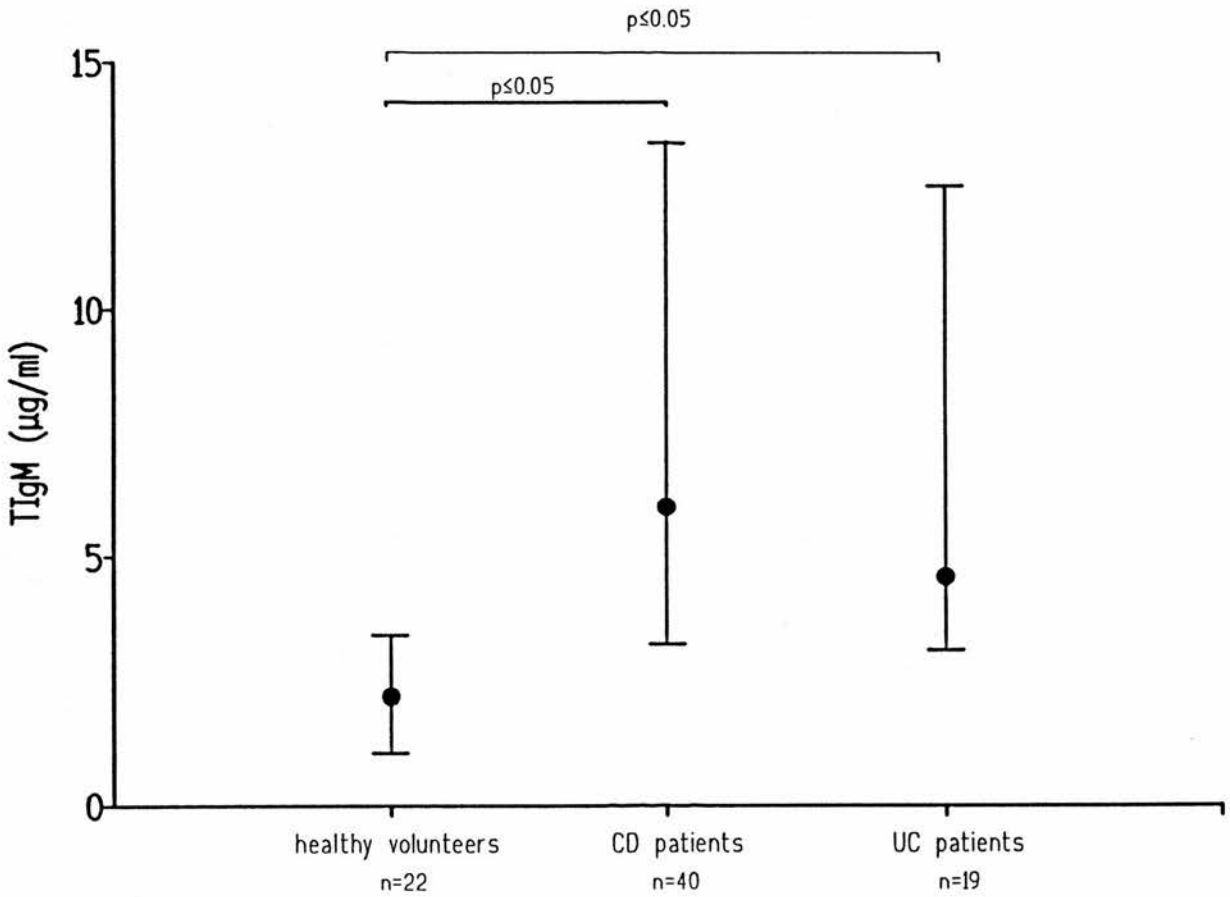


Fig. 5.II. Concentrations of TIgM in WGL fluid of healthy volunteers compared to patients with Crohn's disease (CD) and ulcerative colitis (UC). •=Median. Bars show the upper and lower quartiles of the ranges.

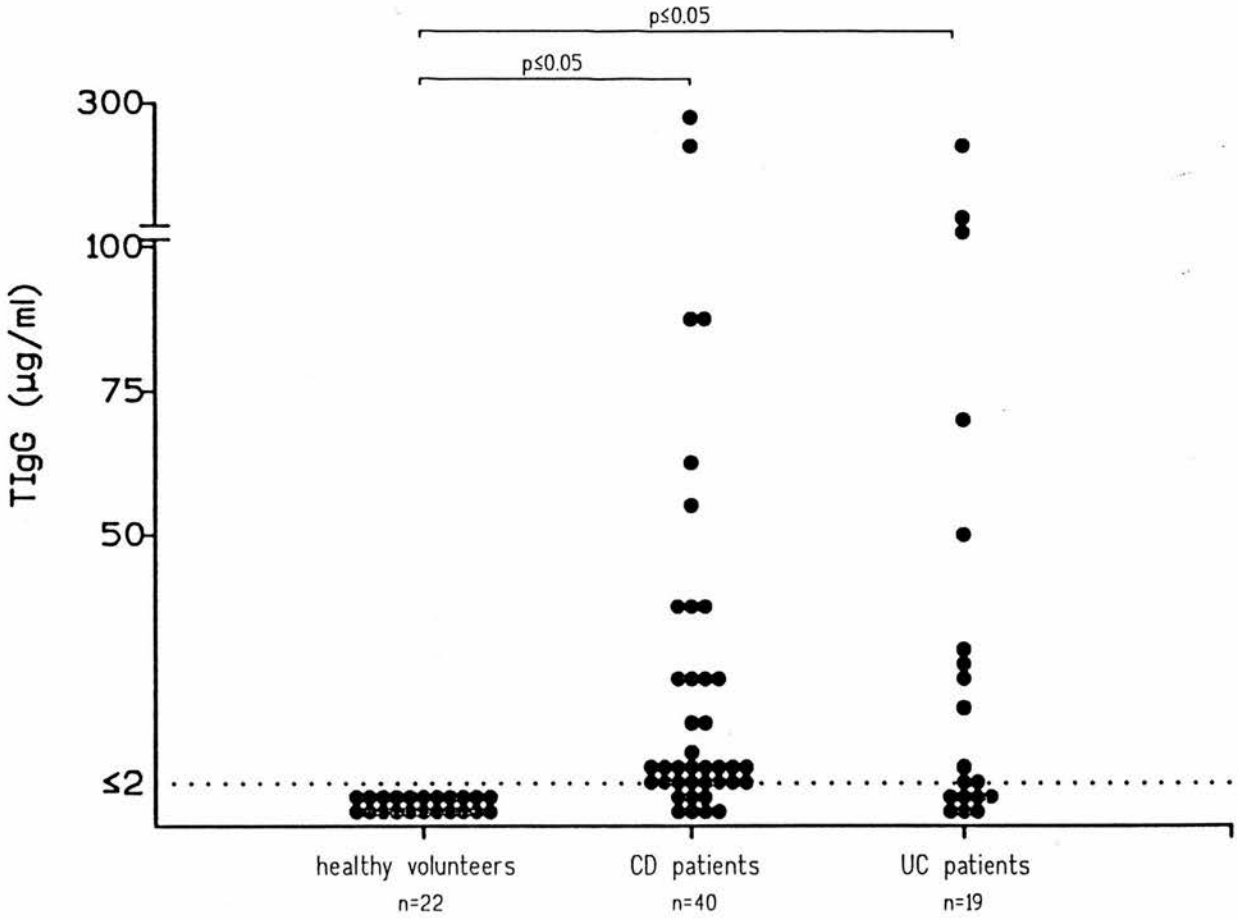


Fig. 5.III. Concentrations of TIgG in WGL fluid of healthy volunteers compared to patients with Crohn's disease (CD) and ulcerative colitis (UC).

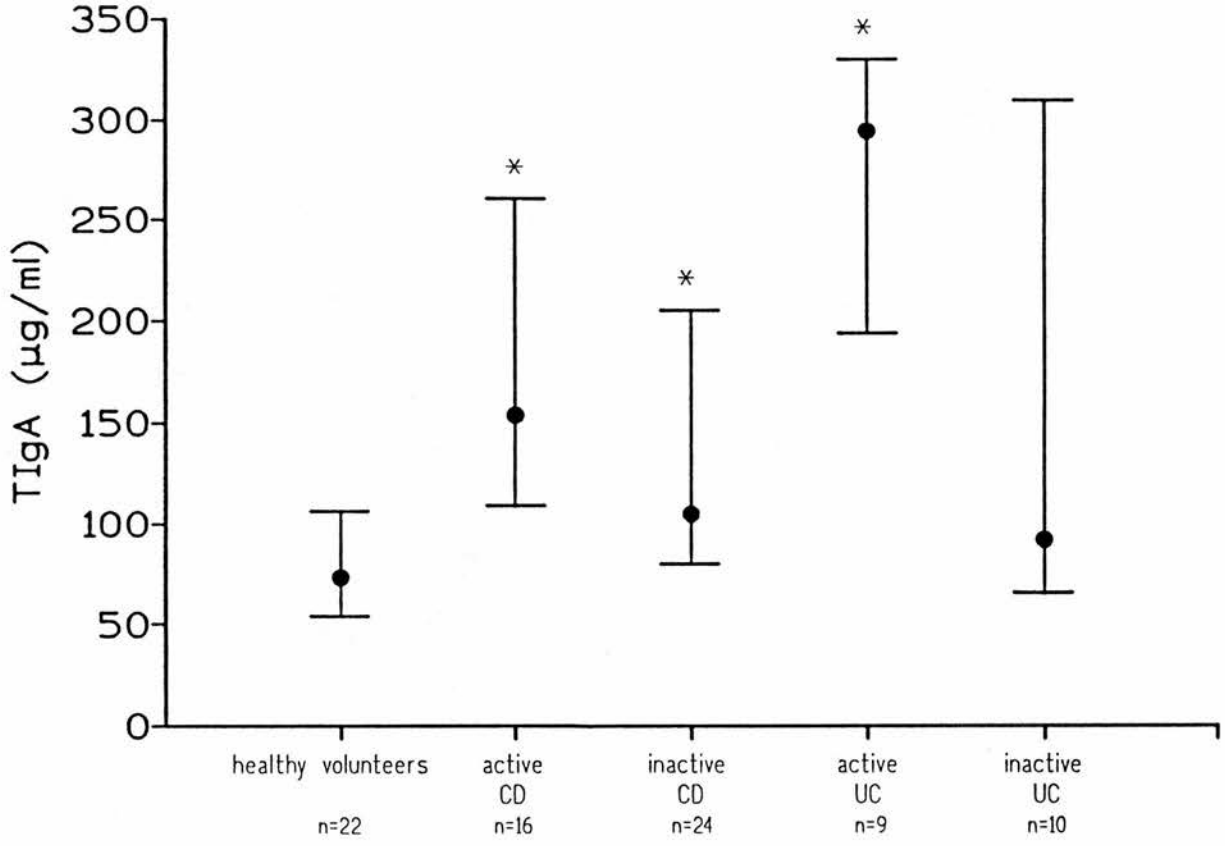


Fig. 5.IV. Concentrations of TIgA in WGL fluid of healthy volunteers compared to active & inactive Crohn's disease (CD) and active & inactive ulcerative colitis (UC). •=median. *=levels are significantly higher than healthy volunteers. Bars show the upper and lower quartiles of the ranges.

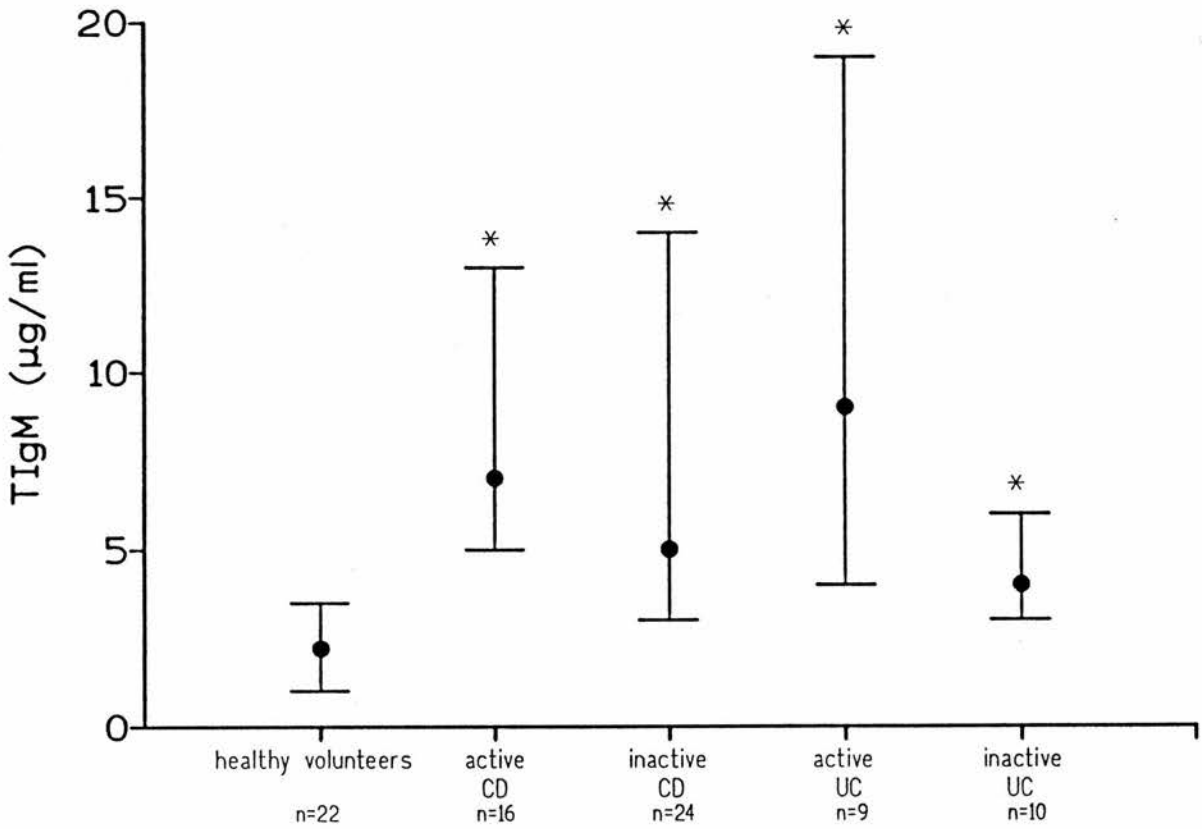


Fig. 5.V. Concentrations of TIgM in WGL fluid of healthy volunteers compared to active & inactive Crohn's disease (CD) and active & inactive ulcerative colitis (UC). •=median. *=levels are significantly higher than in healthy volunteers. Bars show the upper and lower quartiles of the ranges.

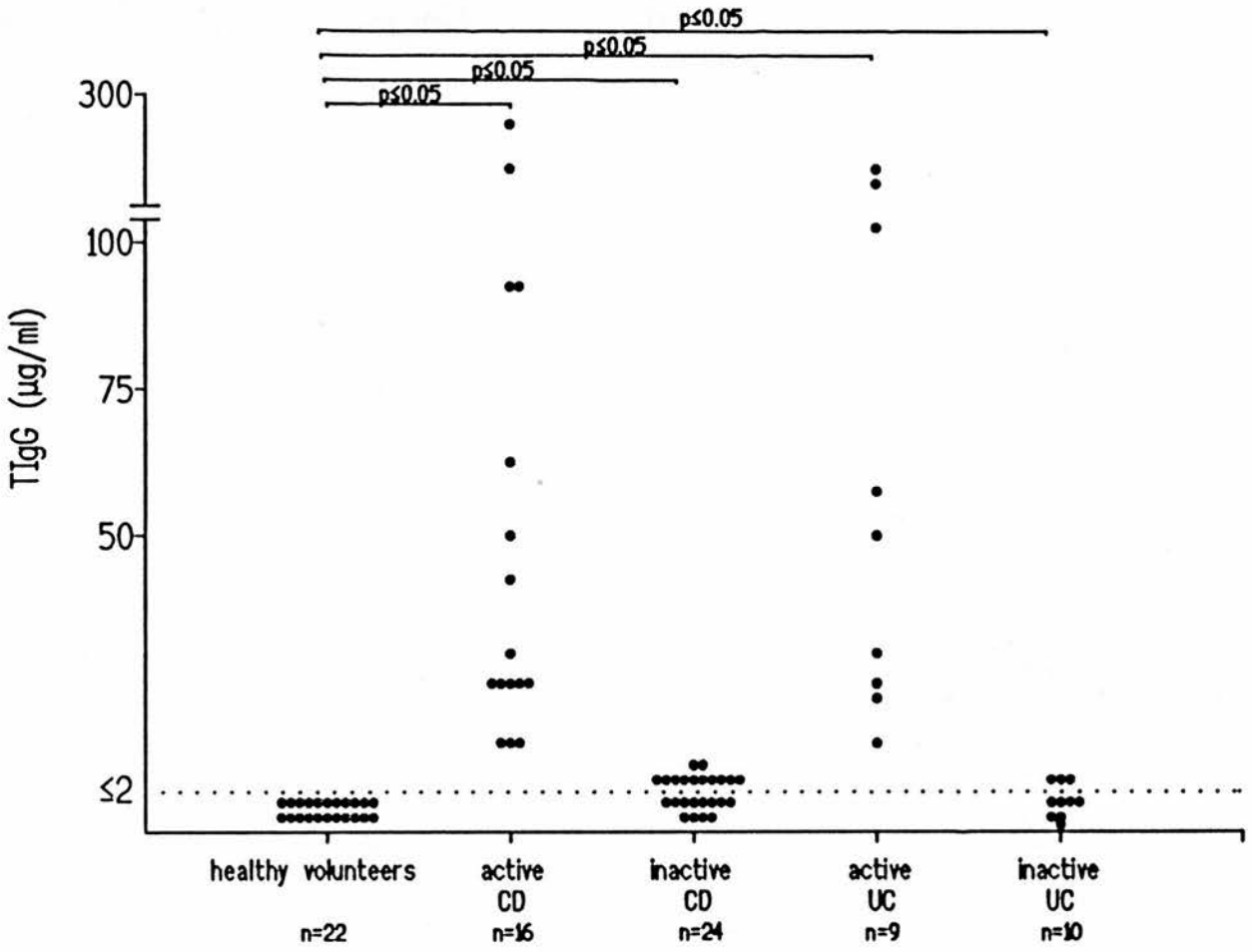


Fig. 5.VI. Concentrations of TIgG in WGL fluid of healthy volunteers compared to active & inactive Crohn's disease (CD) and ulcerative colitis (UC).

Chapter VI

THE INTESTINAL IMMUNE RESPONSES TO THE ORAL TYPHOID VACCINE Ty21a

Introduction

Humans are the only host for *Salmonella typhi* and infection may follow ingestion of contaminated food or water with clinical illness (typhoid fever) developing after an incubation period of 7-14 days. It has been estimated that about 33 million cases of typhoid fever occur annually world-wide (Institute of Medicine, 1986) and although patients with typhoid (as well as paratyphoid) fever are encountered in all parts of the world, they are now primarily found in those countries of the developing world where sanitary conditions and the standards of personal and food hygiene are poor.

In developed countries with good sewerage and water supply systems, most cases of typhoid fever are sporadic and either imported (by travellers or imported food) or can be traced to contact with a chronic carrier (approximately 3% of patients with typhoid fever become chronic carriers) (Hoffman, 1991). In the tropics and subtropics, epidemics occur, with the peak of incidence in the hot and dry months of the year when the concentration of the organisms in the water is higher (not diluted by rain) (Hoffman, 1991). However, the majority of cases of typhoid fever are found in endemic areas where the annual blood culture-positive attack rate may be more than 1000/100,000 population per year (Simanjuntak et al, 1991).

In most endemic areas the incidence of typhoid fever is higher among children who are 5-19 years old (Edelman and Levine, 1986; Ashcroft, 1962) i.e. schoolchildren. This is of potential relevance in the control of typhoid fever since schoolchildren represent an important group in which typhoid fever can be controlled by school-based immunisation programmes.

Vaccination against typhoid fever is particularly important since, as mentioned above, it is an important enteric infection among school-age children in the developing countries. Furthermore, there are increasing numbers of reports about the appearance of multiple drug-resistant *Salmonella* spp. world-wide. Particularly problematic are species resistant to the three main antibiotics which are traditionally used for the treatment of enteric infection; chloramphenicol, ampicillin, and co-trimoxazol (Rowe et al, 1990). The increase in the number of travellers who lack natural immunity against typhoid fever from industrialised countries to areas known to be endemic for typhoid fever has increased the need to develop a vaccine against this enteric infection.

Until recently, protection against typhoid fever could only be provided by parenteral vaccines of heat-killed, phenol- or acetone-preserved whole-cell *Salmonella typhi* organisms. Parenteral killed whole-cell typhoid vaccines have been available since 1896 (Groschel and Hornick, 1981) and have been used throughout this century. Two doses of these vaccines, 4-6 weeks apart, provides significant protection against typhoid fever in endemic areas (varied

from 51 to 88%) among children and adults for around 3 years (Levine et al, 1989). However, local and systemic side effects associated with the administration of these vaccines are common and it has been estimated that between 25 and 50% of the vaccinees will have an adverse reaction following the administration of the vaccine (Hoffman, 1991) in addition to the fact that the above vaccines must be given parenterally. More details about the disadvantages of parenterally-administered vaccines were described in chapter 2.

The above drawbacks of the parenterally-administered killed whole-cell vaccines stimulated intensive research seeking oral vaccines with minimal side effects. As a result, a live attenuated oral typhoid vaccine was developed in the early 1970s by chemical mutagenesis of a pathogenic strain of *Salmonella typhi* with the production of the vaccine strain Ty21a (gal-E mutant strain) (Germanier and Fürer, 1975). The appearance of the vaccine Ty21a offered new hope of an effective oral vaccine without significant side effects. Details of the development of this vaccine were cited in chapters 2 and 3 of this thesis.

The vaccine Ty21a has been demonstrated to be genetically stable, safe and provided a significant protection against experimentally challenged *Salmonella typhi* at a dose of 10^5, a dose that caused typhoid fever in 53% of unimmunised control volunteers (Germanier and Fürer, 1975; Gilman et al, 1977). Field studies into the protective efficacy of the vaccine Ty21a have shown various

degrees of protection against typhoid fever between 42 and 96% (Simanjuntak et al, 1991; Wahdan et al, 1980).

Based on the results of the protective efficacy obtained from the field trials, the vaccine Ty21a has been licensed in USA, Australia and many European countries, including UK, for the prophylaxis against typhoid fever. The recommended dosages, schedules and formulation of the vaccine have varied from country to another. For instance, in the USA four doses of the vaccine Ty21a has been recommended to be taken every other day (Wolfe, 1990) while in the UK only three doses of the vaccine has been recommended to be taken on three alternate days in the form of enteric-coated capsules (Department of Health, 1992).

In spite of the fact that the aim of administering an oral live vaccine, such as Ty21a, is to stimulate specific intestinal antibodies, in particular secretory IgA (SIgA), yet an extensive research work has been directed to field trials measuring the protective efficacy of the vaccine (Wahdan et al, 1980; Levine et al, 1987; Simanjuntak et al, 1991) or the immune response in the serum of the vaccinees (Black et al, 1983). Field trials have assessed the protective efficacy of the vaccine in different areas with different rates of incidence of infection. However, there is no evidence that the vaccine Ty21a provides protection against naturally-acquired infection with typhoid fever in healthy adults from non-endemic areas. Furthermore, as was mentioned in chapter 2, it is now

widely accepted that systemic immunity is quite separate from mucosal immunity and thus the findings in serum cannot be extrapolated to the gut. Forrest et al (1991) investigated the effect of a variety of formulations, dosages and the timing of administration of the vaccine Ty21a in a group of healthy Australian soldiers using jejunal aspirate as a material for the study. However, in another study Forrest (1992a) accepted that obtaining jejunal fluids is an invasive, labour-intensive procedure, which is inconvenient for the patient and the investigator. In addition, the findings in the jejunal aspirate may only reflect the state of the immune response at the level of the jejunum and not the whole gut.

Therefore, investigation of the effect of the oral live typhoid vaccine Ty21a in British healthy volunteers by using WGL technique may provide a better understanding of the kinetics of the immune response against this vaccine in the complex micro-environment of the gut.

Subjects and methods

A total of 25 adult healthy British volunteers (2 females and 23 males) were recruited for this study. Of these volunteers, 22 (2 females and 20 males) completed the experiment successfully. Three volunteers were excluded for a variety of reasons (see below). The median age of the 22 volunteers was 27

with a range of 22-44 years. The volunteers recruited for this study were selected from the records of the Clinical Research Centre of the Department of Medicine, University of Edinburgh. Selection of the volunteers was based on the following criteria: the age, alcohol drinking, and smoking habit. Subjects aged less than 20 or more than 60 years and subjects who drink more than 20 units of alcohol per week were excluded. Only subjects who are either heavy smokers (smoke more than 20 cigarettes per day for the last 3 years or more) or absolutely non-smokers were selected. The reason for selection of heavy smokers and non-smokers in this study was to investigate the effect of smoking on the gut immunity and whether a mitogen such as the oral typhoid vaccine Ty21a would boost an antigen-specific intestinal immune response in this group (see chapter 8).

Volunteers were sent an information sheet explaining the aim of the study, WGL procedure and the preparatory procedures to be followed should the candidate wish to take part in the study. Written consent was obtained in advance from every volunteer. Subjects were asked to attend two sessions of WGL with an interval of 3 weeks between the first and the second session.

Before subjects started drinking the lavage solution on their first visit, 10-20 mls of venous blood samples were taken and an information sheet for every participant was completed. The information sheets contained information about previous vaccination, particularly typhoid vaccine, history of travelling abroad

highlighting visits to third world countries where typhoid fever is known to be endemic, and history of infection especially with *Salmonella typhi* or *Salmonella paratyphi* A, B or C. The information sheet also included records about drug intake, smoking and alcohol drinking habits. In addition, history of previous surgical and/or medical illness, other than infection, was recorded. A copy of the above mentioned information sheet is included in the appendix of this thesis. The procedure of WGL technique proceeded as described in chapter 3.

Three subjects were excluded from the study (all males) due to the following reasons. One volunteer was found, during completion of the information sheet, to be diabetic and on oral hypoglycaemic tablets. Another volunteer was excluded from the study because he failed to complete the first session of WGL because he had nausea one hour after he started drinking the lavage solution. Although the third volunteer had successfully completed the first session of WGL, he had to leave Edinburgh and was not able to attend the second session of the WGL and therefore he was excluded from the study.

After subjects completed the first session of WGL, they were given 3 enteric-coated capsules of the oral typhoid vaccine Ty21a, with each capsule containing 2×10^9 live *Salmonella typhi* organisms, to be taken on days 1, 3 and 5 (day 0 was day of the first WGL session) one hour before or after meal.

Volunteers returned 3 weeks later for the second session of WGL. Another blood sample was taken from every volunteer and further information about any side effect(s) observed following ingestion of the capsules containing the vaccine were recorded before subjects start drinking the lavage solution as described before.

Since results obtained in this study were found to be non-parametric and paired, the Wilcoxon signed-rank test was used for the statistical analysis of the data.

Assays performed

1. ELISA: for the quantitative measurement of total immunoglobulins in processed WGL specimens. Measurement of total immunoglobulins in serum was carried out in the Department of Clinical Biochemistry, WGH.

2. ELISA: for the quantitative measurement of antigen-specific antibodies (anti-*Salmonella typhi* LPS antibodies) both in processed lavage specimens and in serum. The technical details of the above assays have already been described in chapter 3.

3. ELISA: for the quantitative measurement of anti-Rc mutant of *Salmonella typhimurium* antibodies in processed lavage samples. *Salmonella* is a member of the Enterobacteriaceae family which is a large heterogeneous group of Gram-negative bacilli whose natural habitat is the intestinal tract of humans and animals. Therefore, antigens of *Salmonella typhi* may be shared not only by other strains of *Salmonella* species but also by other members of family. Thus, whether the intestinal immune responses stimulated after administration of the oral typhoid vaccine Ty21a were specific to *Salmonella typhi* antigens, or due to cross-reaction with antigens of *Salmonella* species other than *Salmonella typhi*, or antigens of other members of the Enterobacteriaceae family, had to be further investigated. Rc mutant antigen of *Salmonella typhimurium* is a common antigen shared with at least 3 members of the Enterobacteriaceae family (*E. coli*, *Shigella* and *Salmonella*). Therefore, Rc mutant antigen was exploited to confirm that the anti-*Salmonella typhi* LPS immune response induced after administration of the vaccine Ty21a was specific for the *Salmonella typhi* organisms and not due to non-specific immune up-regulation i.e. to exclude the possibility that the increase in the post-vaccination anti-*Salmonella typhi* LPS antibodies were due to polyclonal activating effect of the vaccine Ty21a.

Results

1. **The oral typhoid vaccine Ty21a**: apart from mild abdominal discomfort reported by one volunteer, the oral live typhoid vaccine Ty21a was well-tolerated by all other subjects involved in this study and there were no side effects or complaints about this vaccine.

2. **Total IgA**: the median value of the pre-vaccination total IgA (TIgA) in WGL fluid of 22 volunteers was 73.25 μg/ml with a range of 10.00-173.20 whereas the median of the post-vaccination TIgA was 69.60 μg/ml with a range of 20.00-225.30. There was no significant difference between pre- and post-vaccination TIgA ($p=0.63$). (Fig 6.I)

3. **Total IgM**: the median value of the pre-vaccination total IgM (TIgM) in lavage specimens was 2.20 μg/ml with a range of 0.10-9.50 whereas the median of the post-vaccination TIgM was 1.75 μg/ml with a range of 0.10-5.10. There was no significant difference between pre- and post-vaccination TIgM ($p=0.16$). (Fig. 6.II).

4. **Anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid**: the median of the pre-vaccination IgA in WGL fluid was 57.30 units/mg of TIgA with a range of 12.00-265.10 whereas the median of the post-vaccination IgA was 120.20 units/mg of TIgA with a range of 6.90-1518.60. There was an

overall significant increase in the post-vaccination specific IgA in WGL specimens ($p=0.018$). (Fig. 6.III).

There was a significant increase in specific IgA in lavage specimens of 14 out of 22 volunteers (63.6%) after vaccination with the oral typhoid vaccine Ty21a. However, there was a significant decrease in specific IgA in the lavage specimens of 4 out of 22 volunteers (18.2%). Four volunteers out of 22 were found to have no significant change in specific IgA in their lavage samples.

Results were considered to be significantly increased or decreased if the changes were found to be more than 20% greater or less than the basic values. This is because the inter-assay coefficient of variation of the ELISA used in this assay was about 15-20%.

5. **Anti-*Salmonella typhi* LPS IgM antibodies in WGL fluids**: the median of the pre-vaccination specific IgM in WGL specimens was 0.10 units/ml with a range of 0.10-1.60 whereas the median of the post-vaccination specific IgM was 0.65 with a range of 0.10-12.10 units/ml. There was an overall significant increase in the post-vaccination specific IgM ($p=0.006$). (Fig. 6.IV).

6. **Anti-*Salmonella typhi* LPS IgA antibodies in serum**: the median of the pre-vaccination specific IgA in the serum of 22 volunteers was 407.00

units/ml with a range of 46.00-7150.00 whereas the median of the post-vaccination specific IgA was 727.00 units/ml with a range of 42.00-7274.00. There was an overall significant increase in post-vaccination specific IgA in the serum of 22 healthy volunteers ($p=0.004$). (Fig. 6.V).

7. **Anti-*Salmonella typhi* LPS IgM antibodies in serum**: the median of the pre-vaccination specific IgM in serum was 791.00 units/ml with a range of 277-5540 whereas the median of the post-vaccination specific IgM was 920.00 units/ml with a range of 373.00-6446.00. There was an overall significant increase in the post vaccination specific IgM in the serum of 22 healthy volunteers ($p=0.014$).

8. **Anti-*Salmonella typhi* LPS IgG antibodies in serum**: the median of the pre-vaccination specific IgG in the serum was 511.0 units/ml with a range of 167.0-4499.0 whereas the median of the post-vaccination specific IgG was 720.0 units/ml with a range of 169.0-46267.0. There was an overall significant increase in the post-vaccination specific IgG in the serum of 22 healthy volunteers ($p=0.009$). (Fig. 6.VI).

9. **The correlation between specific antibodies in lavage fluid and serum**: The correlation between anti-*S. typhi* LPS IgA antibodies in WGL fluid and serum before and after vaccination was studied in order to investigate the

relationship, if any, between systemic (serum) and local (WGL fluid) humoral immune responses. There was no association between anti-*S. typhi* LPS IgA antibodies in WGL fluid and serum before or after vaccination ($r=0.077$, $p=0.732$ for the correlation before vaccination; $r=0.255$, $p=0.252$ for the correlation after vaccination) (Fig. 6.VII). The correlation between the increase in specific IgA antibodies in lavage fluid and the increase in specific IgA antibodies in serum after vaccination was also investigated and it was found that there was no association between the changes in serum and those in WGL specimens ($r=0.25$, $p=0.252$). Likewise, it was found that there was no association between the increase in specific IgM antibodies in lavage fluid and serum ($r=0.36$, $p=0.10$).

The correlation between levels of anti-*S. typhi* LPS IgA in WGL fluid and anti-*S. typhi* IgG antibodies in serum before and after vaccination was also investigated and it was found that there was no correlation between the above antibodies ($r=142$, $p=0.527$ for the correlation before vaccination; $r=0175$, $p=0.437$ for the correlation after vaccination) (Fig. 6.VIII). Likewise, the correlation between the increase in specific IgA antibodies in lavage fluid and the increase in specific IgG antibodies in serum was also investigated and it was found that there was no association between changes of specific IgA in lavage fluid specimens and specific IgG antibodies in serum ($r=0.28$, $p=0.20$).

10. **Anti-Rc mutant of *Salmonella typhimurium* IgA antibodies in WGL fluids**: the median of the pre-vaccination anti-Rc mutant IgA antibodies was found to be 166.30 units/ml with a range of 2.00-1324.60 whereas the median of post-vaccination anti-Rc mutant IgA antibodies was 242.30 units/ml with a range of 38.10-1206.20. There was no significant increase in the post vaccination anti-Rc mutant IgA antibodies ($p=0.127$). (Fig. 6.IX).

Conclusion

The live oral typhoid vaccine Ty21a was well-tolerated, safe and produced minimal, if any, side effects. This vaccine, given at 3 doses (each dose contains 2×10^9 live *Salmonella typhi* organisms) over 3 alternate days in the form of enteric-coated capsules, significantly increased specific intestinal IgA antibodies in 14 out of 22 (63.6%) adult healthy British volunteers. Although there was an overall increase in the anti-*Salmonella typhi* LPS antibodies in the serum of the volunteers involved in this study, yet the statistical tests carried out to investigate the relationship between the increase in the quantity of specific antibodies in serum and those in lavage fluid have clearly demonstrated that there is no association between systemic and intestinal humoral immune responses to the oral typhoid vaccine Ty21a.

The indirect ELISA method used for the quantitative measurement of anti-*Salmonella typhi* LPS antibodies to investigate the effect of the oral typhoid vaccine on gut immunity was shown to be specific. The anti-*Salmonella typhi* LPS antibodies due to a cross-reacting antigen(s) were not significantly increased.

I also concluded that the technique of WGL is non-invasive, safe and well-tolerated by most of the volunteers. Furthermore, WGL is a reliable method for obtaining material to study gut immunity.

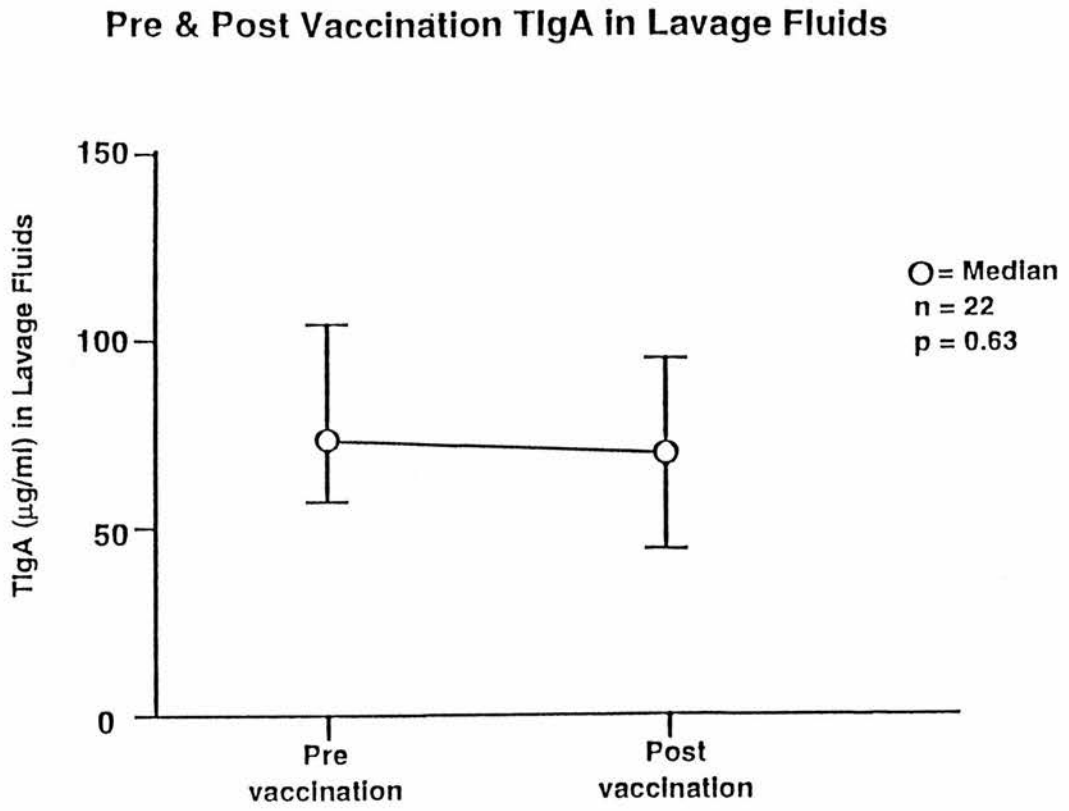


Fig. 6.I. Pre- & post-vaccination TIgA in WGL fluid of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity. Bars show the upper and lower quartiles of the ranges.

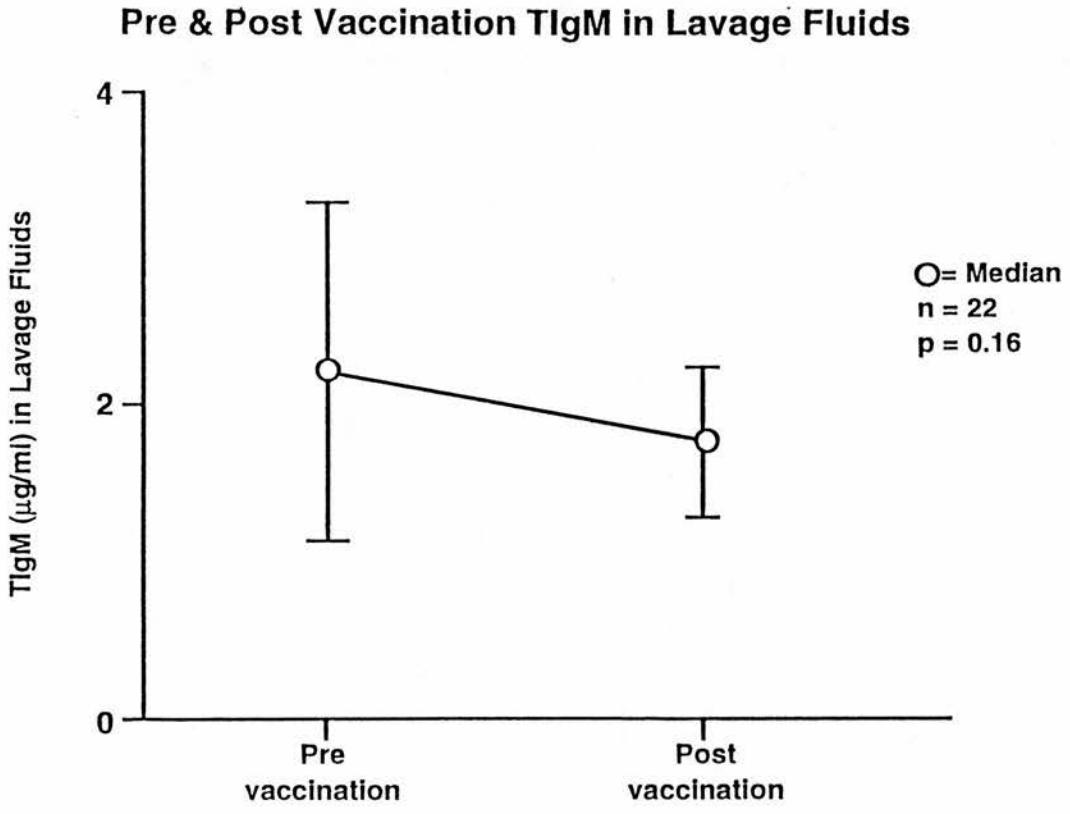


Fig. 6.II. Pre- & post-vaccination TIgM in WGL fluid of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity. Bars show the upper and lower quartiles of the ranges.

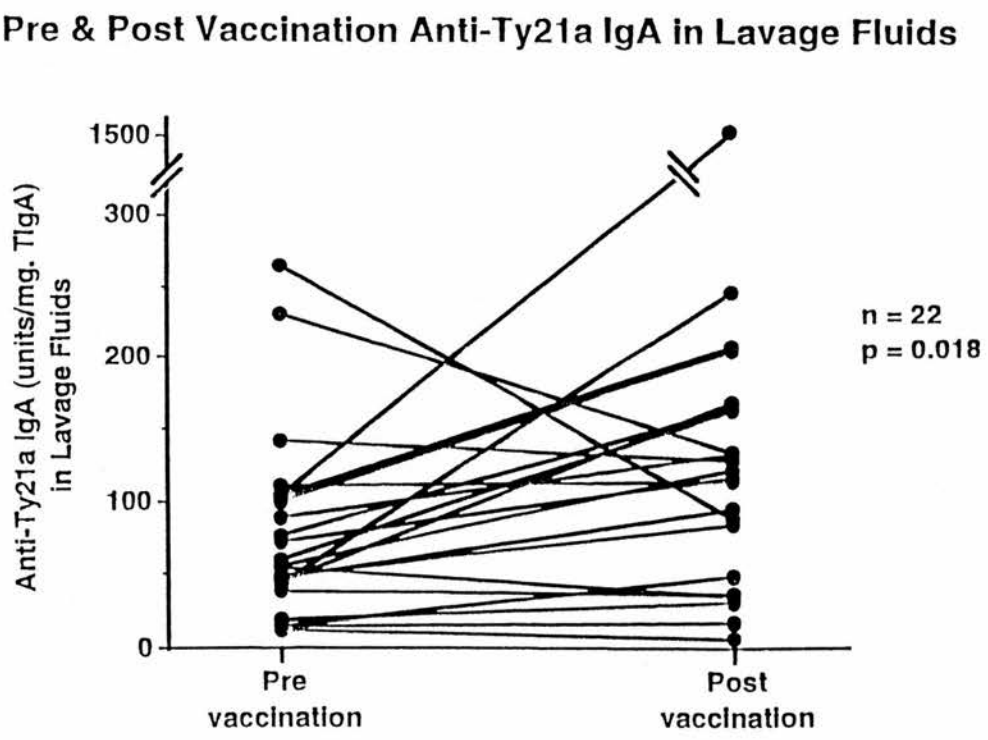


Fig. 6.III. Pre- & post-vaccination anti-*S. typhi* LPS IgA antibodies in WGL fluid of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity.

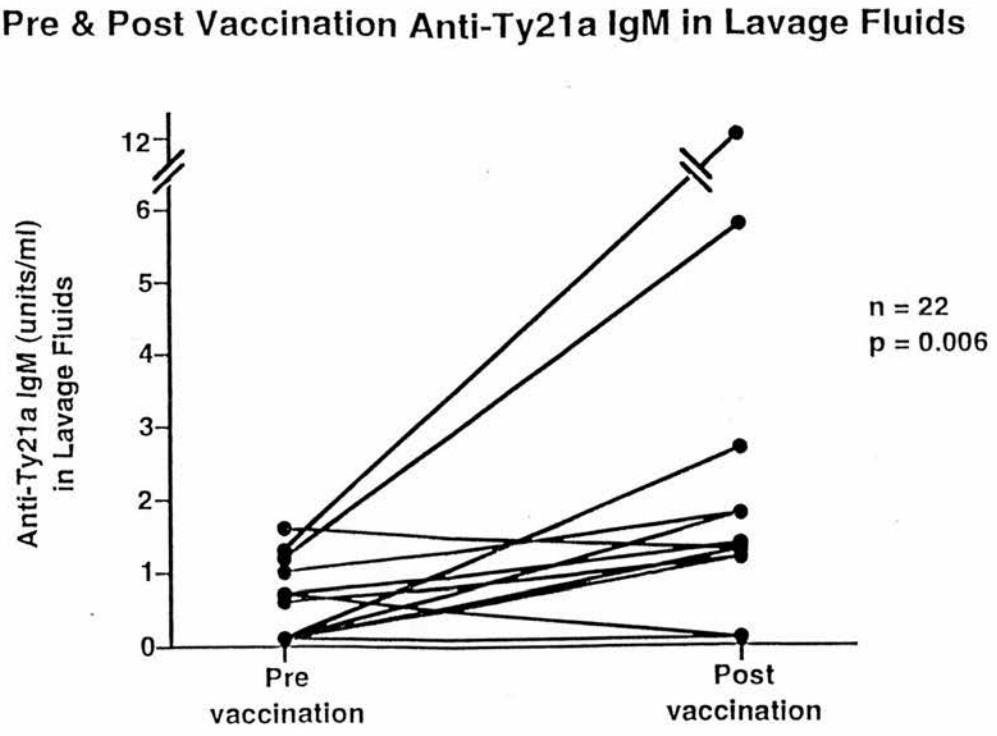


Fig. 6.IV. Pre & post-vaccination anti-*S typhi* LPS IgM antibodies in WGL fluid of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity.

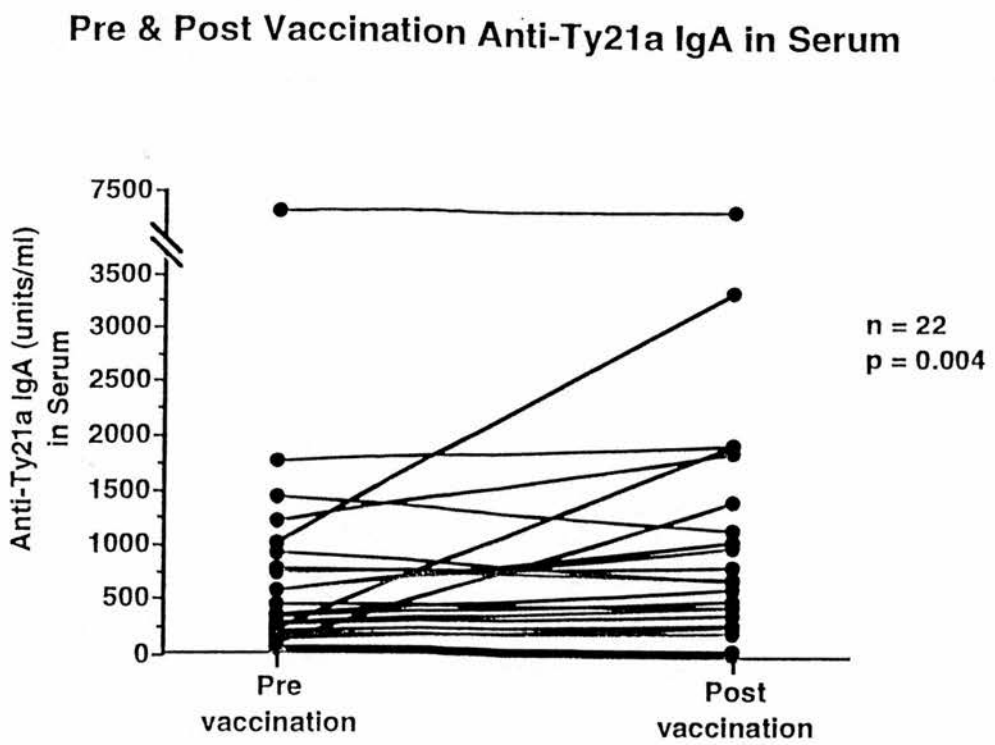


Fig. 6.V. Pre- & post-vaccination anti-*S. typhi* LPS IgA antibodies in serum of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity.

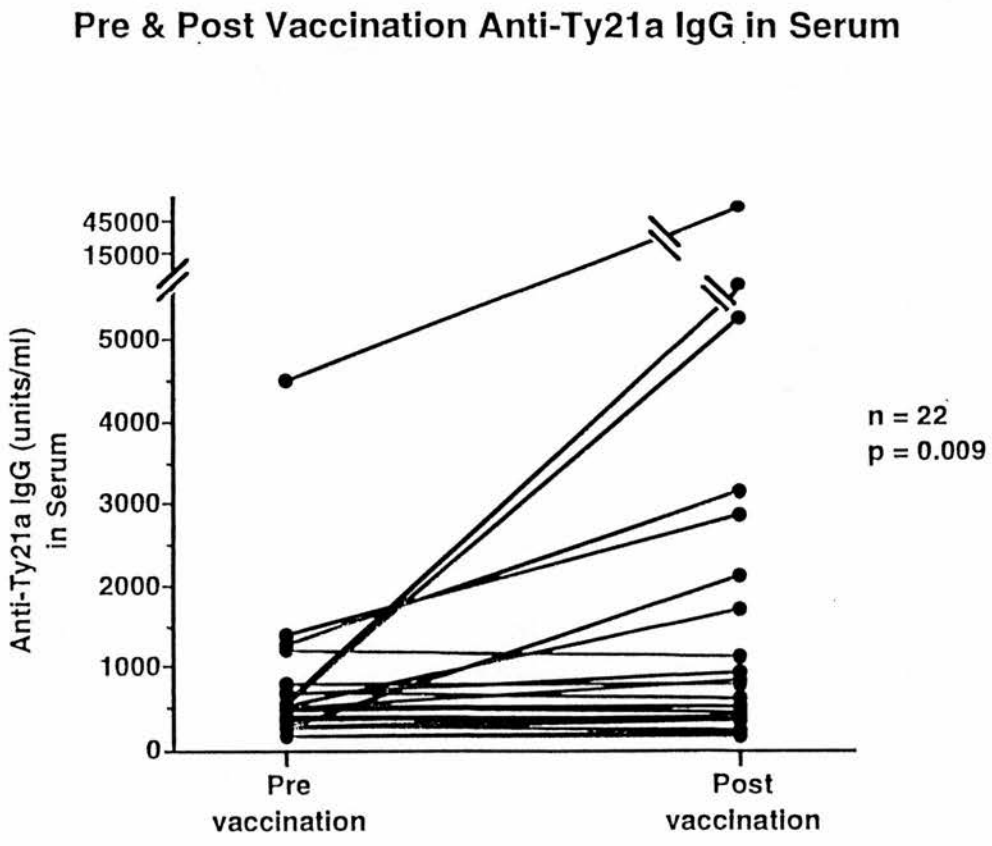


Fig. 6.VI. Pre- & post-vaccination anti-*S. typhi* LPS IgG antibodies in serum of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity.

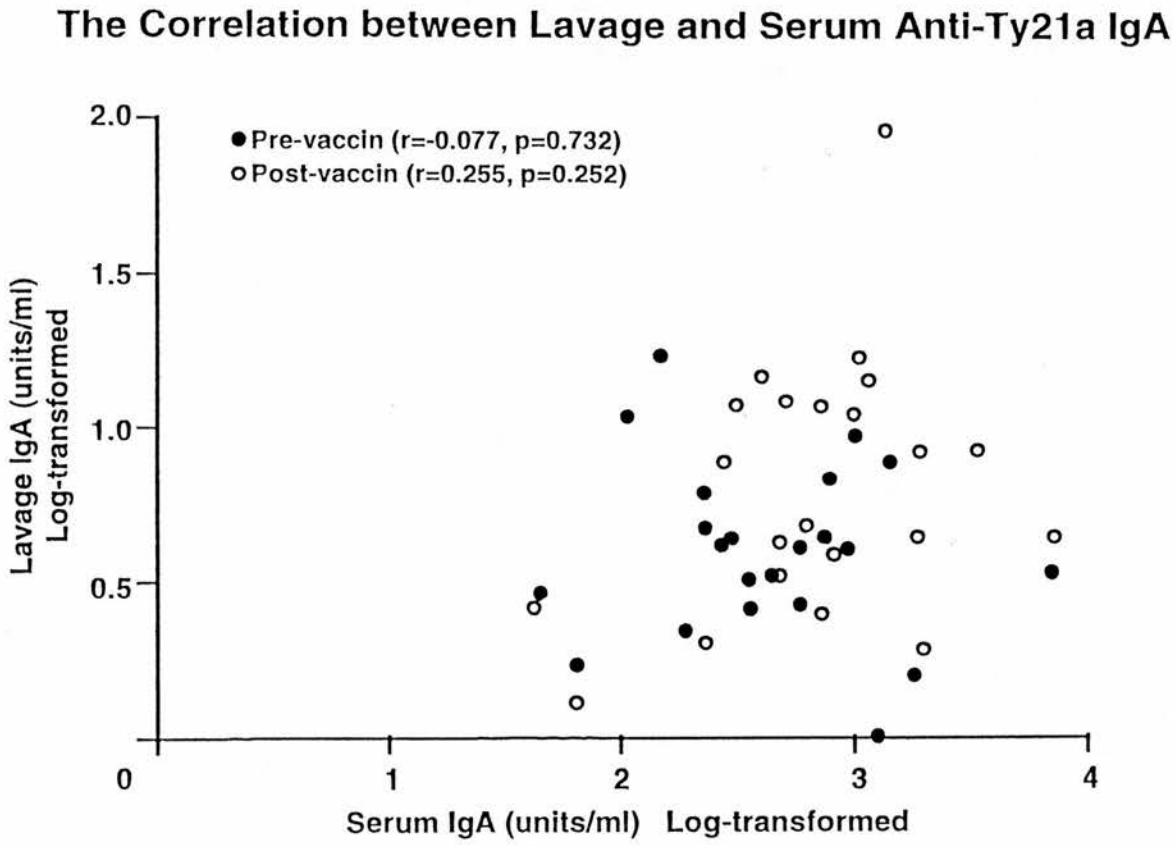


Fig. 6.VII. The correlation between concentrations of anti-*S. typhi* LPS IgA antibodies in WGL fluid and serum, before and after vaccination, of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity.

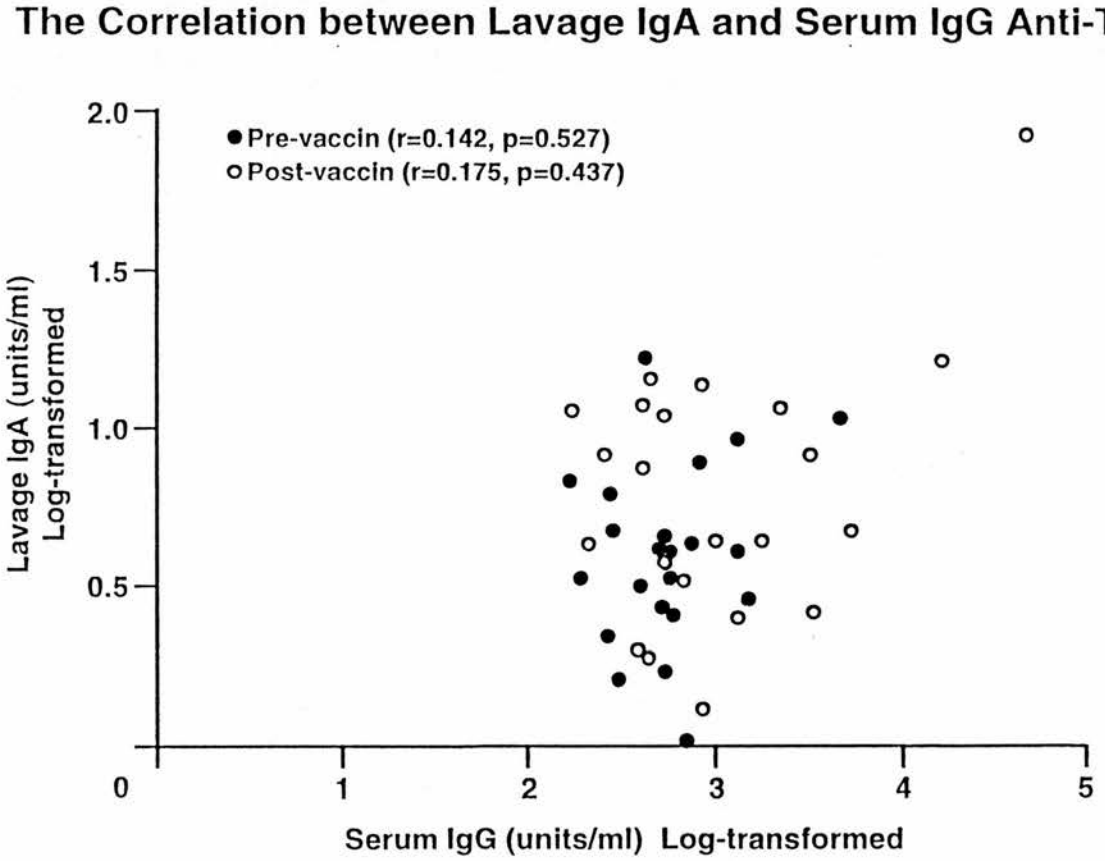


Fig. 6.VIII. The correlation between concentrations of anti-*S. typhi* LPS IgA antibodies in WGL fluid and anti-*S. typhi* LPS IgG antibodies in serum, before and after vaccination, of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity.

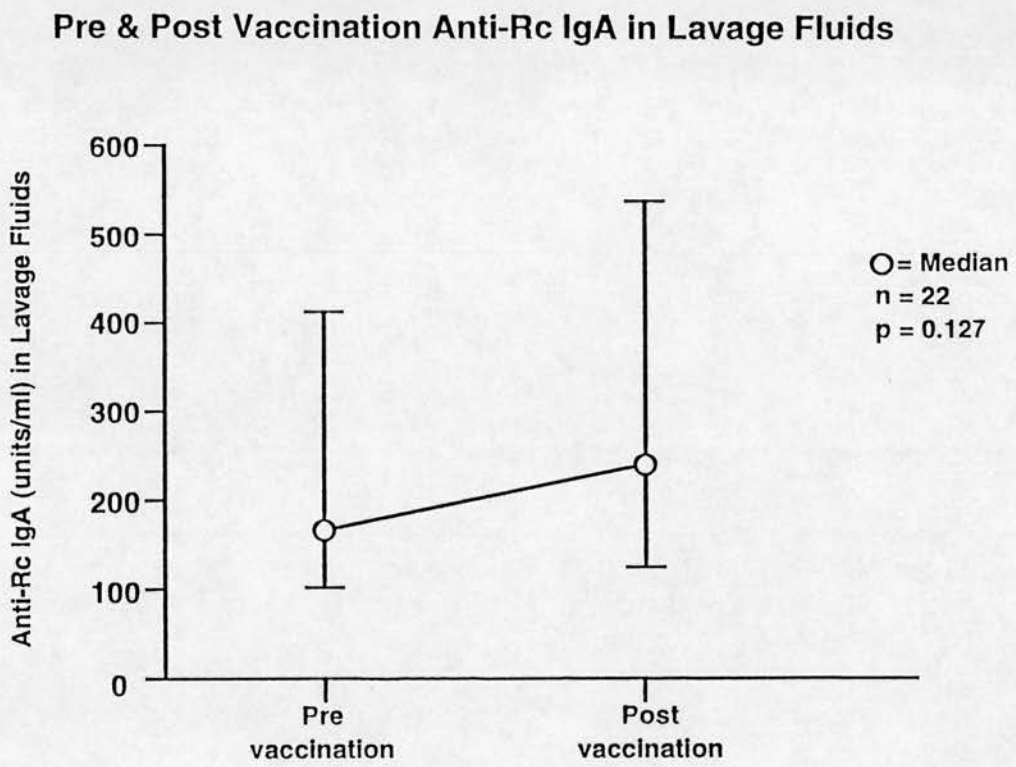


Fig. 6.IX. Pre- & post-vaccination anti-*S. typhimurium* Rc-mutant IgA antibodies in WGL fluid of 22 British healthy volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity. Bars show the upper and lower quartiles of the ranges.

Chapter VII

THE INTESTINAL IMMUNE RESPONSES TO NATURALLY-ACQUIRED SALMONELLA INFECTION

Historical background

The genus *Salmonella* was so named in honour of Daniel Elmer Salmon, a veterinary pathologist who was the director of the Bureau of Animal Industry in Washington DC, where the organism *Salmonella cholerae-suis* was discovered by his assistant Theobald Smith in 1885 (Grady and Keusch, 1971). *Salmonella cholerae-suis* was thought, incorrectly, by Smith, to be the causative organism of the swine cholera (a viral disease).

Microbiology

Salmonella is a genus of the family Enterobacteriaceae, a large heterogeneous group of Gram-negative bacilli. *Salmonella* spp. are non-capsulated, non-sporulating aerobic bacilli that usually ferment glucose, mannitol and maltose, but not lactose, producing acid and gas (with the exception of *Salmonella typhi* which produces acid but not gas). With the exception of *Salmonella gallinarum* and *Salmonella pullorum*, all *Salmonella* spp. are almost always motile by peritrichous flagella which may be expressed in two or more antigenic forms (H antigens).

In addition to the flagellar (H) antigens, *Salmonella* spp., like other members of the family Enterobacteriaceae, possess one or more somatic (O) antigens on which the Kauffmann-White classification of *Salmonella* spp. is based (Kauffmann,

1969). These are due to lipopolysaccharide (LPS) structure and have been grouped A to Z with additional numbered categories. An additional somatic, heat-labile antigen known as Vi (virulence) antigen (also known as the capsular or K antigen) is found in *Salmonella typhi* and *Salmonella paratyphi* C and prevents agglutination with anti-O antisera. Virulence of particular strain of *Salmonella* in mice was found to be positively correlated with the amount of Vi antigen possibly due to the fact that the capsule provides additional protection from phagocytosis (see chapter 2). However, some strains of *Salmonella typhi* that lack the Vi antigens can cause disease in man (Griffiths and Gorbach, 1993).

The Kauffmann-White classification of *Salmonella* spp. includes more than 2000 combinations of O and H antigens and although pathogenic *Salmonella* organisms are grouped A to Z, more than 90% of the human pathogenic salmonellae fall into groups A to E which contain 40 serotypes.

An alternative approach to the Kauffmann-White classification of *Salmonella* spp., is the classification based on the host specificity (Candy and Stephen, 1988). According to this classification, *Salmonella* spp. are divided into 3 main categories: *Salmonella typhi* (one serotype) infecting only man; *Salmonella cholerae-suis* (one serotype) infecting swine; and *Salmonella enteritidis* (approximately 1700 serotypes) which may infect humans or animals.

Salmonella spp. are found in the intestinal tract of many vertebrates and in the internal organs and bloodstream of some invertebrates. *Salmonella* organisms have been found in whales, tortoises, house flies, sheep, rats, chicken, buffalo etc. (Griffiths and Gorbach, 1993). They are also found in rivers, sea water and sewage (Snydman and Gorbach, 1991). Salmonellae cause major food-born diseases on a global basis of both personal and economic importance world-wide (Rodrigue et al, 1990). Although the incidence of typhoid fever has declined in many developed countries, the incidence of non-typhoidal salmonellosis may actually be rising (Longfield, 1991). In the USA, it has been estimated that there are approximately 0.8-3.7 million cases of food poisoning due to *Salmonella* each year (Chalker and Blaser, 1988).

Contaminated food is understandably the most frequent vehicle for transmission of *Salmonella* organisms. Outbreaks are often traced to commercially processed meat and meat products, inadequately cooked poultry and unpasteurized milk or dairy products. In the USA, pets have been implicated in about 3% of outbreaks of salmonellosis (Centres for Disease Control, 1986). Other routes of transmission include flies and other insects. Recently, gastrointestinal endoscopy has also been found to be responsible for transmission of *Salmonella* organisms (Longfield, 1991).

There are many factors which make understanding of the pathogenesis and immunopathology of salmonella infection an extremely difficult task. In

addition to the fact that a large number of *Salmonella* serotypes are responsible for human diseases, there appear to be a variety of potential virulence factors among the various serotypes. Vi antigen and the outer membrane LPS are two independent virulence factors and it has been found that different strains of *Salmonella* organisms are virulent at different infective doses (Carter et al, 1987). For instance, *Salmonella pullorum*, a chicken pathogen, was found to be unable to produce diseases in humans at a dose of 10^9 while only 10^5 *Salmonella newport* were found to cause overt diseases in some volunteers.

In humans, salmonellae produce a wide range of diseases which varies from asymptomatic carrier through a self-limited enteritis to typhoid fever (see below). The important pathogenic event associated with salmonella infection is the invasion of the epithelium of the terminal ileum and colon (Keusch, 1991) which has been suggested to occur through M cells (Sneller and Strober, 1986). The possible relationship between gut pathogens and M cells was described in chapter 2.

Clinical syndromes associated with salmonella infection include the following. (a) Gastroenteritis: this accounts for about 75% of documented salmonella infection and is characterised by nausea, vomiting, abdominal pain followed by diarrhoea which may last from 3 to 5 days. Diarrhoea varies from minimal to dysenteric with purulent and bloody stools and even cholera-like diarrhoea (Black et al, 1960). The latter type of diarrhoea is most frequently seen in individuals with achlorhydria (Gray and Trueman, 1971). (b) Bacteraemia: this is found in about 10% of cases.

It varies from transient bacteraemia accompanying some cases of gastroenteritis to bacteraemia which may result in lethal shock (Griffiths and Gorbach, 1993). (c) Localised Infection: this accounts for about 5% of salmonella infection and may occur in any organ following bacteraemia resulting in endocarditis, meningitis, arteritis, osteomyelitis etc. (Black et al, 1960). There is a well-known association between sickle cell anaemia and salmonella osteomyelitis. (d) Enteric fever: this is caused by *Salmonella typhi* (causes typhoid fever) and *Salmonella paratyphi* A, *Salmonella paratyphi* B and *Salmonella paratyphi* C (cause paratyphoid fever). Typhoidal salmonella infection differs from non-typhoidal infection in being primarily a systemic and not intestinal illness. Typhoid and paratyphoid fever remain a serious health problem in the developing countries. Typhoid fever has been described in chapter 6. (e) Chronic asymptomatic carrier state: if *Salmonella* organisms continue to persist for more than one year, the patient is regarded as a chronic carrier. The carrier state may follow both typhoidal or non-typhoidal infection, after either symptomatic or asymptomatic infection. It has been estimated that about 0.2-0.6% of non-typhoidal and 1-3% of typhoidal *Salmonella* cases will become chronic carriers (Musher and Rubenstein, 1973; Kaye, 1991).

Mechanisms of diarrhoea due to salmonella infection

The precise mechanism(s) of diarrhoea associated with salmonella infection is (are) not yet well-understood. However, three mechanisms have been suggested for the

diarrhoea due to salmonella infection. (a) *Salmonella* organisms may produce toxins (enterotoxins) which stimulate fluid secretion into the gut lumen. Enterotoxins produced by *Shigella* organisms (Shiga toxins) are known to cause secretion of fluid into gut lumen resulting in accumulation of large amount of fluid and causing diarrhoea (Keusch et al, 1972). (b) Invasion of the gut epithelium by *Salmonella* organisms results in interaction with polymorphonuclear cells that then release prostaglandins at the site of invasion thereby causing diarrhoea (Gorbach, 1988). (c) Damaged epithelium may be unable to reabsorb fluids from the gut lumen resulting in accumulation of fluid in the gut lumen and diarrhoea (Griffiths and Gorbach, 1993).

The intestinal immune responses against bacterial infection of the gut

In addition to a number of non-specific defence mechanisms by which the intestine is protected from colonisation by bacteria (see chapter 2), there are specific immune mechanisms that provide additional protection of the gut. These are the humoral immune responses and cell-mediated immunity.

Intestinal humoral immune responses. Bacterial infection of the gastrointestinal tract normally produces an intestinal secretory antigen-specific immune responses, usually SIgA (Chan et al, 1981). The unique structure of SIgA renders this antibody well-suited to protect the intestinal mucosal surface (see chapter 2 for the

structure and function of SIgA). SIgA antibodies have been reported to appear within one week of the onset of bacterial infection and within as little as 3 days after re-immunisation (Svennerholm et al, 1984a). In addition to the function of immune exclusion known to be performed by SIgA, it has recently been shown that SIgA mediates antibody-dependent T cell-mediated cytotoxicity (Tagliabue et al, 1984) and interferes with the utilisation, by bacteria, of necessary growth factors such as iron in the intestinal environment (Holmgren and Svennerholm, 1992). Furthermore, it has been suggested that SIgA may play a role in the inductive stage of the mucosal immune responses by facilitating antigen uptake by Peyer's patches (Holmgren and Svennerholm, 1992).

Cell-mediated immune responses. Cell mediated mucosal immune reactions through major histocompatibility complex (MHC)-restricted cellular cytotoxicity, natural killer (NK) cell activity and antibody-dependent cellular cytotoxicity have been shown to play an important role in the intestinal mucosal immunity against invasive enteric infection caused by *Salmonella* organisms (Tagliabue et al, 1984). Interferon gamma (IFN-γ)-producing T cells have been reported to be significantly increased in number in the human duodenal mucosa after intestinal antigenic challenge and it has been suggested that these cells may play an important role in the intestinal mucosal immune defence (Tagliabue et al, 1992). IFN-γ has been shown to increase IgA production, MHC antigen expression and the expression of the secretory-component receptors on the enterocytes surfaces (Brandtzaeg et al, 1990). Furthermore, IFN-γ has been found to interfere with the tight junctions

between intestinal epithelial cells, *in vitro*, such that permeability is increased. It also interfere with the receptivity of epithelial cells to invasive enteric pathogens (Holmgren et al, 1989).

Generally, intestinal infections with *Salmonella* seem to prime the intestinal immune system. Responses elicited by salmonella infection (as well as other enteric pathogens) vary and depend, to a large extent, on the virulence factors of the pathogen, mode of the reaction by the host, and the degree of the invasiveness of the pathogen. Although serum antibodies are commonly detected even after non-invasive intestinal infections (Levine and Nataro, 1994), they are not believed to play an important role in the protection against gut infections.

Aim of the study

The aim of this study was to investigate the intestinal humoral immune responses to naturally-acquired salmonella infection, both typhoidal and non-typhoidal. Patients who had had salmonella infection in the preceding 12 months were recruited for the study. Concentrations of total immunoglobulins and antigen-specific antibodies in WGL fluid of those patients were measured and compared with the levels of immunoglobulins and antibodies in WGL fluid of healthy British volunteers and IBD patients. Levels of immunoglobulins and antigen-specific antibodies in the sera of the above patients and volunteers were also studied and compared.

The British healthy volunteers (or at least some of them) may have had salmonella infection in the form of salmonella food poisoning, gastroenteritis and/or enterocolitis, particularly those who had a history of travelling abroad. Therefore, it was important to examine levels of antibodies in the gut of these volunteers as an indicator of probable previous exposure to salmonella infection.

Patients with IBD are known to have general up-regulation of the intestinal mucosal immunity with increased production of intestinal IgG, IgM and IgA (Schreiber et al, 1992). Therefore, investigating levels of anti-*Salmonella typhi* LPS antibodies in WGL fluid of patients with IBD and comparing them with other immunologically normal subjects may provide valuable information regarding the intestinal antigen-specific immune responses.

Patients and Methods

Eight patients (4 females and 4 males) who had had naturally acquired salmonella infection were recruited for this study. The mean age of these patients was 41.25 (±12.34 SD). Six out of 8 patients had the manifestations of salmonella infection during or soon after returning from holidays abroad. The rest of the patients had the infection while in the UK. Patients were recruited from the out-patient department of the City Hospital as well as from the Day Bed Area of the Endoscopy Theatre of the Western General Hospital. Patients who had had

salmonella infection (either typhoidal or non-typhoidal), treated and discharged from the City hospital within the preceding 12 months were recruited. Likewise, patients who were referred to Endoscopy Theatre for a variety of gastrointestinal diseases and had recovered from salmonella infection within the preceding 12 months were also recruited for this study. During their illness, patients were treated with ciprofloxacin (4-quinolone) or had had only supportive therapy. At the time of the study, none of the recruits was taking antibiotics and all patients' stools were culture-negative for salmonella organisms. The serotypes of salmonella organisms involved in the infection and other details are given in table 7.I. Apart from the history of salmonella infection, all patients were fit and well and had no history of immunological disorders.

patient	age	sex	date of infection	date of lavage	*Salmonella* serotypes
1	23	F	21/08/91	21/03/92	*S. paratyphi* A
2	23	M	21/06/92	03/09/92	*S. typhi*
3	39	M	17/04/92	23/11/92	*S. typhi*
4	57	M	10/10/90	22/09/91	*S. enteritidis*
5	47	F	19/07/91	17/10/91	*S. enteritidis*
6	49	F	15/10/91	09/01/92	*S. enteritidis*
7	44	F	23/07/91	24/02/92	*S. enteritidis*
8	48	M	26/07/92	08/12/92	*S. enteritidis*

Table 7.I. Patients involved in the study of intestinal immunity following naturally-acquired salmonella infection.

All recruits underwent WGL. The protocol and methodology of WGL were as described in chapter 3. Blood samples were also obtained from all patients at the

time of the study. ELISA was used to measure levels of TIgA and TIgM in WGL fluids and also for quantitative measurement of anti-*Salmonella typhi* LPS specific IgA, IgM and IgG antibodies in WGL fluids and in the sera of all patients. The technical details of ELISA methodology were as described in chapter 3. The concentrations of total IgA, IgM and IgG in the sera of patients were measured by the Department of Clinical Biochemistry.

Since IgA is the predominant immunoglobulin in the intestinal secretions, anti-*Salmonella typhi* LPS IgA in WGL fluid were expressed in two different ways: (a) units/ml (an arbitrary value of 1000 units of anti-*Salmonella typhi* LPS antibodies per ml was designated to the concentration of the standard reference serum, see chapter 3); (b) units/mg of TIgA. The results obtained in (a) were further calculated considering the concentration of TIgA in WGL fluid expressed as μg/ml. The results of anti-*Salmonella typhi* LPS IgA antibodies expressed as units/mg of TIgA were used in the comparison between patients who had had salmonella infection, healthy volunteers and IBD patients.

Results of the concentration of anti-*Salmonella typhi* LPS IgM antibodies in WGL fluid, however, were found to be too low to be expressed as units/mg of TIgM and therefore, they were expressed as units/ml.

Levels of immunoglobulins and antigen-specific antibodies in WGL fluid and sera of the patients involved in this study were compared with immunoglobulins and

antibodies in WGL fluid and sera obtained from the 22 healthy British volunteers described in chapter 6. The data of the healthy volunteers mentioned in this chapter were the pre-vaccination data. The concentrations of immunoglobulins and antibodies of the above patients were also compared with immunoglobulins and antigen specific antibodies in WGL fluid and sera of 30 patients with inflammatory bowel disease selected from the records of the G.I laboratory, Western General Hospital. These patients were studied during 1990 and 1991 by a colleague who investigated the profiles of immunoglobulins and antibodies in WGL fluid and sera of patients with inflammatory bowel diseases as part of his Ph.D degree (Mwantembe, 1992).

Since all data were found to be non-parametrically distributed, the Mann-Whitney U-test was used for the statistical analysis of the results.

Results

Sections 1-5 below are the results of measurements carried out for samples obtained from the group of 8 patients who had had naturally-acquired salmonella infections. The results are also summarised in table 7.II.

1. **Concentration of TIgA and TIgM in WGL fluid**: the median value of the concentrations of TIgA of patients who had had *Salmonella* infection (typhoidal

and non-typhoidal) was 101.6 μg/ml with a range of 24.5-175.6. The median value of the concentrations of TIgM of those patients was 5.4 μg/ml with a range of 2.3-16.8. Surprisingly, one patient who had had typhoid fever (patient 3) was found to have a concentration of TIgA of 24.5 μg/ml i.e. less than half of the minimum value of the range of the concentrations of TIgA of the rest of the patients (the range of TIgA values of the rest of patients was 57.6-175.6 μg/ml).

2. **Concentration of anti-*Salmonella typhi* LPS antibodies in WGL fluid**: the median value of the concentrations of anti-*Salmonella typhi* LPS IgA antibodies was found to be 5.45 units/ml with a range of 2.10-25.40. When the results were expressed as units/mg of TIgA, the median was found to be 81.2 units/mg of TIgA with a range of 30.1-176.0. The median value of the concentrations of anti-*Salmonella typhi* LPS IgM antibodies was 0.55 units/ml with a range of 0.1-4.0.

3. **Concentration of total IgA, IgM, and IgG immunoglobulins in serum**: the median values of the levels of IgA, IgM, and IgG in the sera of the patients involved in the study were 1741.0, 1166.0 and 12180.0 μg/ml with ranges of 1200.0-4174.0, 887.0-2166.0 and 5829.0-18096.0 for IgA, IgM and IgG respectively.

4. **Concentration of anti-*Salmonella typhi* LPS IgA, IgM, and IgG antibodies in serum**: the median value of the concentrations of IgA, IgM, and IgG antibodies in the sera of the patients were 732.0, 3286.0, and 4708.0 units/ml with ranges of 118.0-3794.0, 631.0-7792.0, and 1270.0-14065.0 for IgA, IgM, and IgG antibodies respectively.

5. **The correlation between anti-*Salmonella typhi* LPS antibodies in WGL fluid and serum**: the correlation co-efficient (r) of the relationship between levels of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid and serum was found to be 0.51 ($p=0.29$) and r value for the relationship between IgM antibodies in WGL fluid and serum was found to be 0.39 ($p=0.35$). The correlation co-efficient of the relationship between anti-*Salmonella typhi* LPS IgA in WGL fluid and IgG antibodies in the serum was found to be zero.

Sections 6-10 are the results of the comparative study between patients who had had salmonella infection, the British healthy volunteers and IBD patients.

6. **Levels of TIgA in WGL fluid of patients who had had salmonella infection compared to TIgA levels in WGL fluid of healthy volunteers and IBD patients**: there was no significant difference between levels of TIgA in WGL fluid of patients who had had salmonella infection and TIgA levels in WGL fluid of healthy volunteers ($p=0.336$). However, levels of TIgA in WGL fluid from patients who had had salmonella infection were found to be significantly

lower than levels of TIgA in WGL fluid of IBD patients (p=0.0003). (Fig. 7.I).

7. **Levels of TIgM in WGL fluid of patients who had had salmonella infection compared to levels of TIgM in WGL fluid of healthy volunteers and IBD patients**: levels of TIgM in WGL fluid from patients who had had salmonella infection were found to be significantly higher than levels of TIgM in WGL obtained from the healthy British volunteers (p=0.029). On the other hand, there were no significant differences between levels of TIgM of the patients who had had salmonella infection and levels of TIgM of the or IBD patients (p=0.986). (Fig. 7.II).

8. **Levels of anti-*Salmonella typhi* LPS IgA and IgM antibodies in WGL fluid of patients who had had salmonella infection compared to levels of IgA and IgM antibodies in WGL fluid of healthy volunteers and IBD patients**: there were no significant differences between levels of anti-*Salmonella typhi* LPS IgA antibodies (expressed as units/mg of TIgA or as units/ml) in WGL fluid of the patients who had had salmonella infection and these antibodies in WGL fluid of healthy volunteers or IBD patients (p values when results were expressed as units/mg of TIgA were 0.523 and 0.06 respectively; p value when results were expressed as units/ml were 0.223 and 0.163 respectively). Likewise, there were no significant differences between the concentrations of IgM antibodies in WGL fluid of the patients who had had salmonella infection and these

antibodies in WGL fluid of healthy volunteers and IBD patients (p values were 0.153 and 0.567 respectively). (Fig. 7.III and Fig. 7.IV).

9. **Comparison between levels of IgA, IgM, and IgG in serum of patients who had had salmonella infection and healthy volunteers**: levels of IgM in the sera of patients who had had salmonella infections were found to be significantly higher than levels of IgM in the sera of the healthy volunteers ($p=0.037$) (Fig. 7.V). However, there were no significant differences between levels of IgA or IgG in the sera of patients who had had salmonella infection and levels of the same immunoglobulins in the sera of the healthy volunteers (p values were 0.869 and 0.250 respectively).

10. **Levels of anti-*Salmonella typhi* LPS IgA, IgM, and IgG antibodies in serum of patients who had had salmonella infection compared to levels of those antibodies in serum of healthy volunteers and IBD patients**: levels of IgG antibodies in the sera of patients who had had salmonella infection were found to be significantly higher than levels of IgG antibodies in the sera of both healthy volunteers and IBD patients (p values were 0.0001 and 0.0002 respectively) (Fig. 7.VI). Levels of IgM antibodies in the sera of patients who had had salmonella infection were found to be significantly higher than levels of IgM antibodies in the sera of the healthy volunteers ($p=0.010$) but not significantly different from levels of IgM antibodies in the sera of IBD patients ($p=0.400$) (Fig. 7.VII). Levels of IgA antibodies in the sera of patients who

had had salmonella infection were not significantly different from levels of IgA antibodies in the sera of both healthy volunteers or IBD patients (p values were 0.181 and 0.245 respectively).

After the results of the 8 patients who had had salmonella infection, as one group, were analysed and compared with results of healthy volunteers and IBD patients, the results of the patients who had had salmonella infection were analysed according to the serotype of *Salmonella* spp. and date of infection. Table 7.IV contains a description of the results of the patients who had had salmonella infection analysed according to the serotype of *Salmonella* spp., antibiotics, if any, prescribed at the time of infection, time since infection (period between time of infection and time of lavage study), anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid (expressed both as units/ml and units/mg of TIgA), anti-*Salmonella typhi* LPS IgM antibodies in WGL fluid and anti-*Salmonella typhi* IgG antibodies in serum.

Tables 7.II and 7.III summarise the results of the immunoglobulins and anti-*Salmonella typhi* LPS antibodies in WGL fluid and in sera respectively, of patients who had had salmonella infection, healthy volunteers and IBD patients.

Conclusion

Levels of TIgA as well as anti-*Salmonella typhi* LPS IgA in WGL fluid of patients who had had naturally-acquired salmonella infection (typhoidal and non-typhoidal) within the preceding 12 months did not significantly differ from levels of those immunoglobulins and antibodies in WGL fluid of the British healthy volunteers while levels of TIgA in WGL fluid of the above patients were found to be significantly lower than levels of TIgA in WGL fluid of IBD patients. However, levels of TIgM in WGL fluid of the *Salmonella*-infected patients were found to be significantly higher than levels of TIgM in WGL fluid of the healthy volunteers while no significant differences were found between levels of anti-*Salmonella typhi* LPS IgM in WGL fluid of patients who had had salmonella infection and the other groups.

Surprisingly, one patient who had had *Salmonella typhi* infection had the lowest concentration of TIgA and anti-*Salmonella typhi* LPS IgA antibodies (expressed as units/ml) in WGL fluid and had low anti-*Salmonella typhi* LPS IgG antibodies in serum.

In serum, only the level of TIgM of patients who had had salmonella infection showed a significant difference (higher) from the level of TIgM of the healthy volunteers. Both IgM and IgG anti-*Salmonella typhi* LPS antibodies in sera of patients who had had salmonella infection were found to be significantly higher

than levels of these antibodies in the sera of the healthy volunteers while levels of IgA antibodies in the sera of the salmonella patients and the healthy volunteers did not show significant differences. No correlation was found between antigen-specific intestinal and systemic immune responses among the patients who had had salmonella infection.

The above results indicate that interpretation of the intestinal (as well as the systemic) immune responses against enteric infection is considerably a difficult task and should be taken into consideration while interpreting the immune responses to oral vaccines against these pathogens. The role of intestinal immune responses produced by various enteric pathogens in protection against subsequent infection is yet to be investigated.

immunoglobulins		*Salmonella* patients	healthy volunteers	IBD patients
TIgA	median	101.6 μg/ml	73.25 μg/ml	227.0 μg/ml
	(range)	(24.5-175.6)	(10.00-173.20)	(101.0-1575.0)
TIgM	median	5.5 μg/ml	2.2 μg/ml	6.5 μg/ml
	(range)	(2.3-16.8)	(0.1-9.5)	(0.1-59.0)
anti-*Salmonella typhi* LPS antibodies				
IgA	median	81.2 units/mg of TIgA	57.3 units/mg of TIgA	41.0 units/mg of TIgA
	(range)	(30.1-176.0)	(12.0-265.1)	(8.7-279.3)
IgM	median	0.55 units/ml	0.1 units/ml	0.10 units/ml
	(range)	(0.1-4.0)	(0.1-1.6)	(0.1-14.7)

Table 7.II. Immunoglobulins and anti-*Salmonella typhi* LPS antibodies in **WGL** fluid of patients who had had salmonella infection, healthy volunteers and IBD patients.

immunoglobulins		*Salmonella* patients	healthy volunteers	IBD patients
IgA	median	1741.0 μg/ml	2011.0 μg/ml	2737.0 μg/ml
	(range)	(1200.0-4174.0)	(558.0-5492.0)	(1166.0-6692.4)
IgM	median	1166.0 μg/ml	861.0 μg/ml	1157.1μg/ml
	(range)	(887.0-2166.0)	(83.0-2436.0)	(269.7-3497.4)
IgG	median	12180.0 μg/ml	9657.0 μg/ml	10788.0 μg/ml
	(range)	(5829.0-18096.0)	(5394.0-14877.0)	6177.0-20358.0)
anti-*Salmonella typhi* LPS antibodies				
IgA	median	732.0 units/ml	407.0 units/ml	1321 units/ml
	(range)	(118.0-3794.0)	(46.0-7150.0)	(287.0-14043.0)
IgM	median	3286.0 units/ml	791.0 units/ml	1638 units/ml
	(range)	(631.0-7792.0)	(277.0-5540.0)	(240.0-20922.0)
IgG	median	4708.0 units/ml	511.0 units/ml	321 units/ml
	(range)	(1270.0-14065.0)	(167.0-4499.0)	(1.0-12602.0)

Table 7.III. Immunoglobulins and anti-*Salmonella typhi* LPS antibodies in **serum** of patients who had had salmonella infection, healthy volunteers and IBD patients.

patients	*Salmonella* serotypes	antibiotics given	time since infection	IgA (in WGL fluid)		IgM (in WGL fluid)	IgG (in serum)
				units/ml	units/mg of TIgA	units/ml	units/ml
1	*S. paratyphi* A	ciprofloxacin	7 months	4.9	76.8	0.2	14064.8
2	*S. typhi*	ciprofloxacin	3 months	6.0	40.2	0.9	14006.0
3	*S. typhi*	ciprofloxacin	7 months	2.1	85.7	0.1	2177.5
4	*S. enteritidis*	nil	11 months	25.4	176.0	4.0	6039.6
5	*S. enteritidis*	nil	3 months	2.8	30.1	0.1	3376.0
6	*S. enteritidis*	ciprofloxacin	3 months	21.6	123.0	1.7	2664.8
7	*S. enteritidis*	nil	7 months	3.4	59.0	1.2	13974.4
8	*S. enteritidis*	nil	5 months	10.4	94.4	0.1	1270.5

Table 7.IV. Results of the 8 patients who had had salmonella infection analysed according to *Salmonella* serotypes and dates of infection.

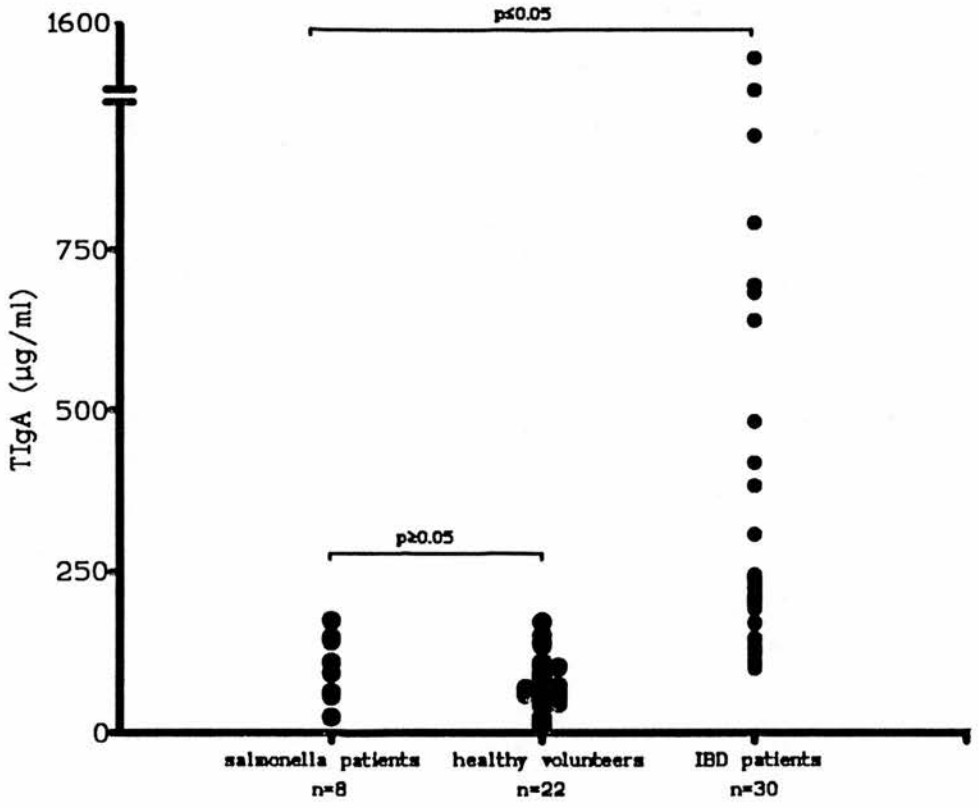


Fig. 7.I. Concentrations of TIgA in WGL fluid of patients who had had salmonella infection as compared to healthy volunteers and IBD patients.

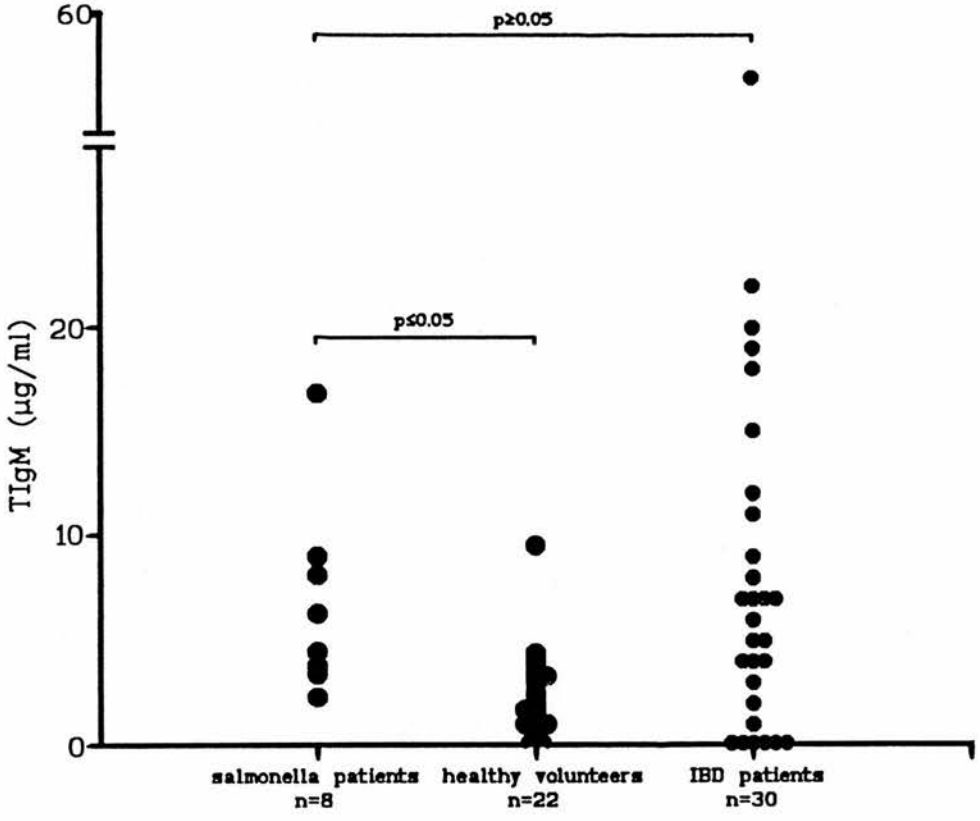


Fig. 7.II. Concentrations of TIgM in WGL fluid of patients who had had salmonella infection as compared to healthy volunteers and IBD patients.

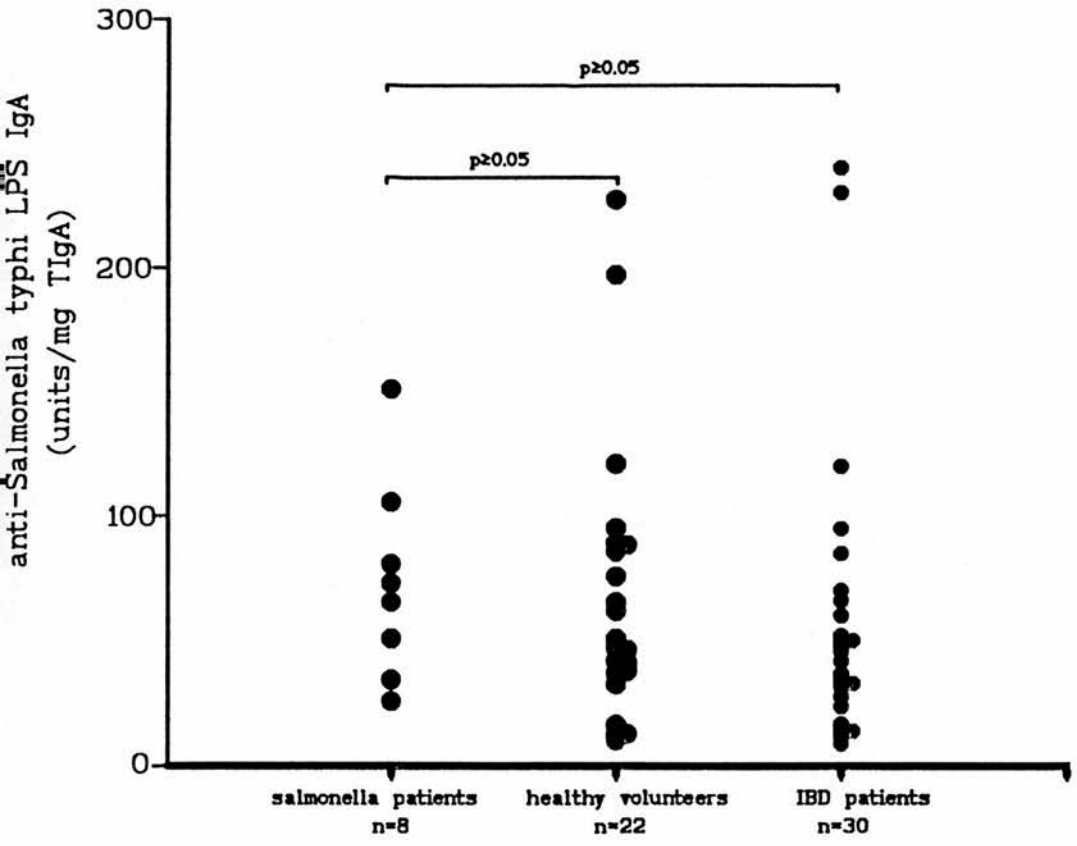


Fig. 7.III. Concentrations of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of patients who had had salmonella infection as compared to healthy volunteers and IBD patients.

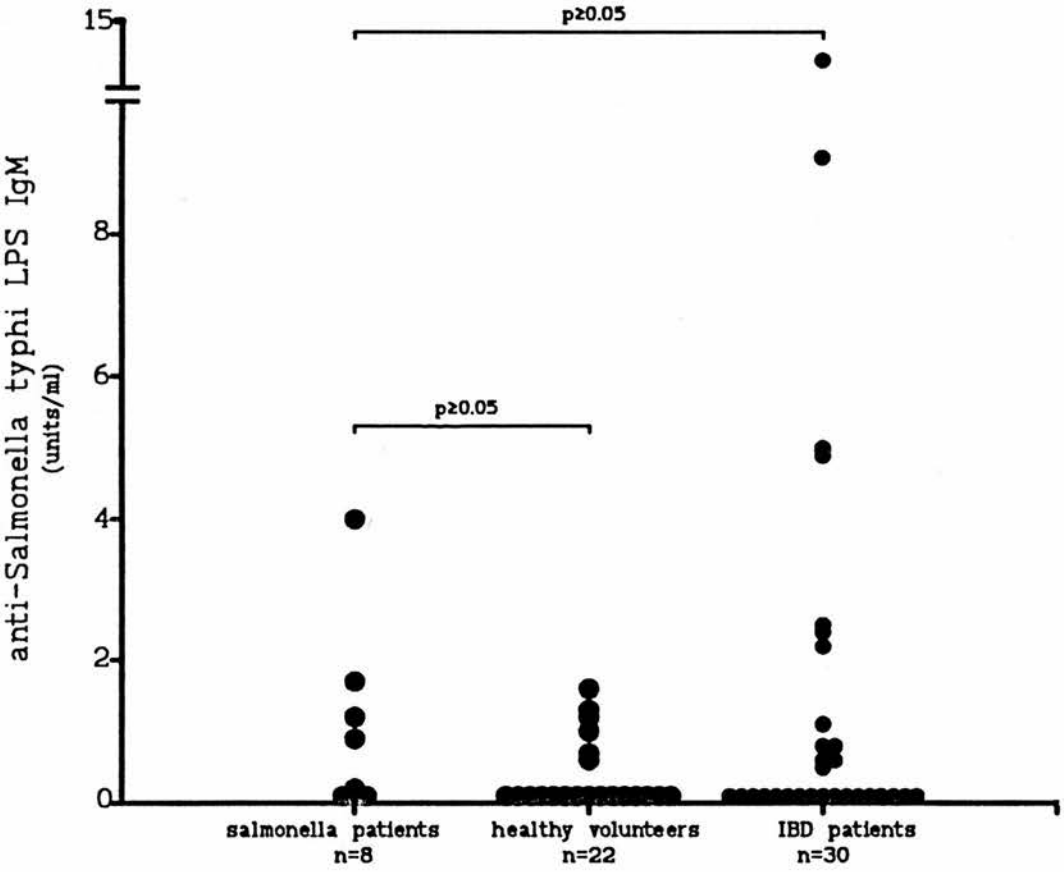


Fig. 7.IV. Concentrations of anti-*Salmonella typhi* LPS IgM antibodies in WGL fluid of patients who had had salmonella infection as compared to healthy volunteers and IBD patients.

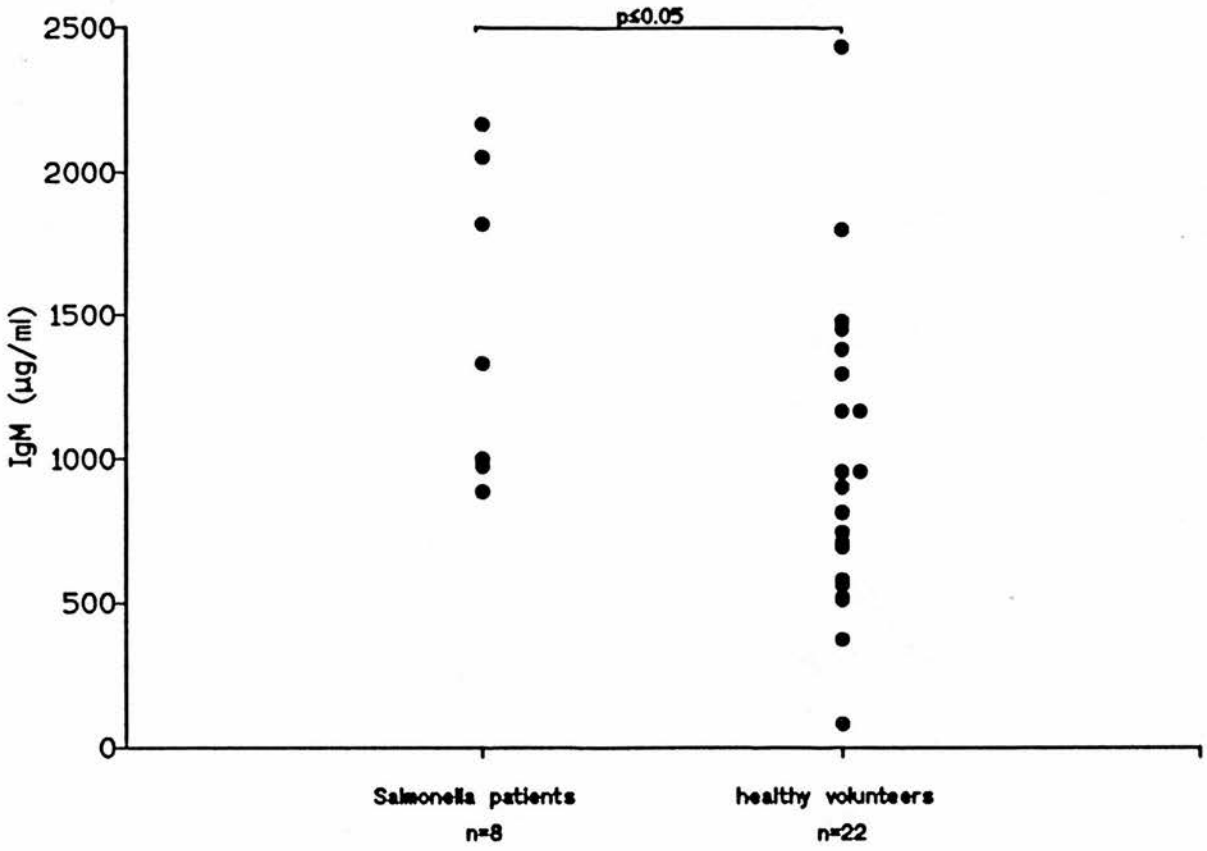


Fig. 7.V. Concentrations of TIgM in sera of patients who had had salmonella infection as compared to healthy volunteers.

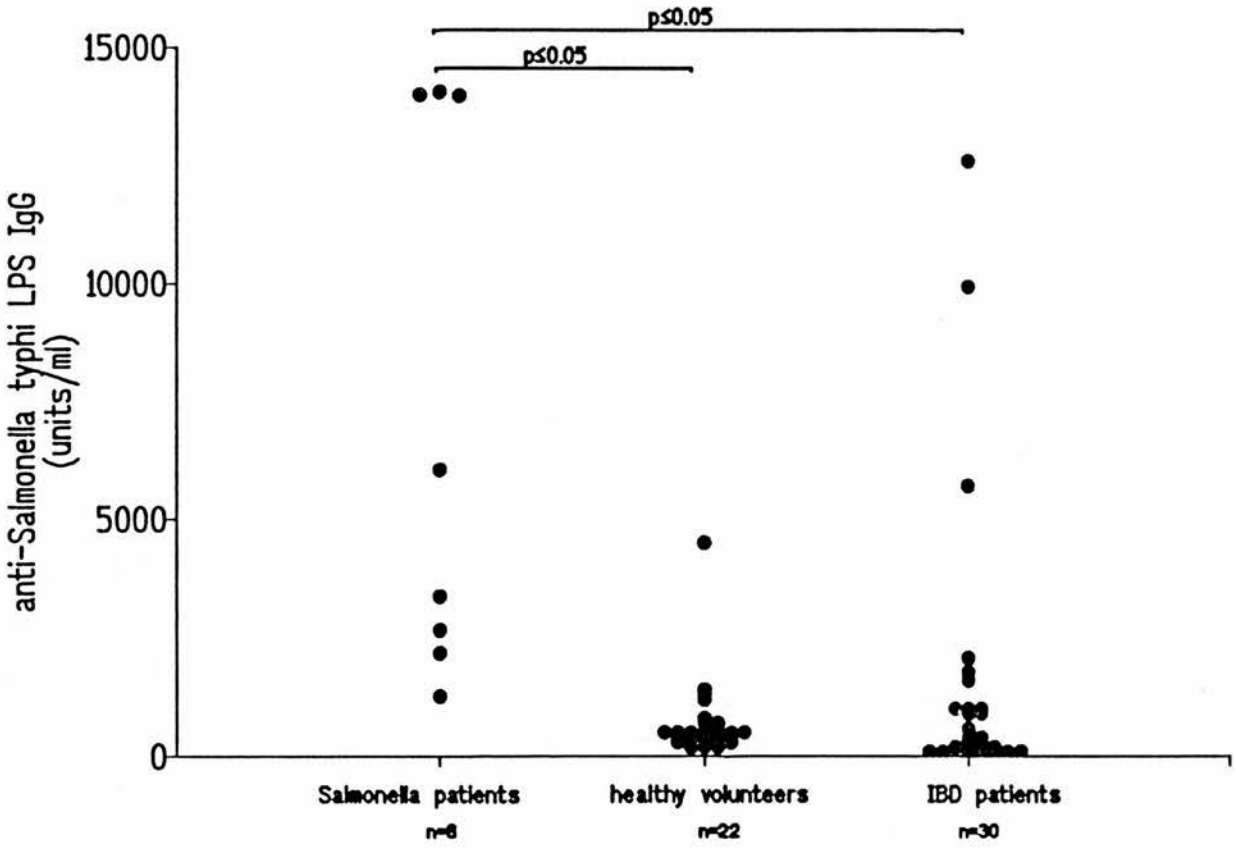


Fig. 7.VI. Concentrations of anti-*Salmonella typhi* LPS IgG antibodies in sera of patients who had had salmonella infection as compared to healthy volunteers and IBD patients.

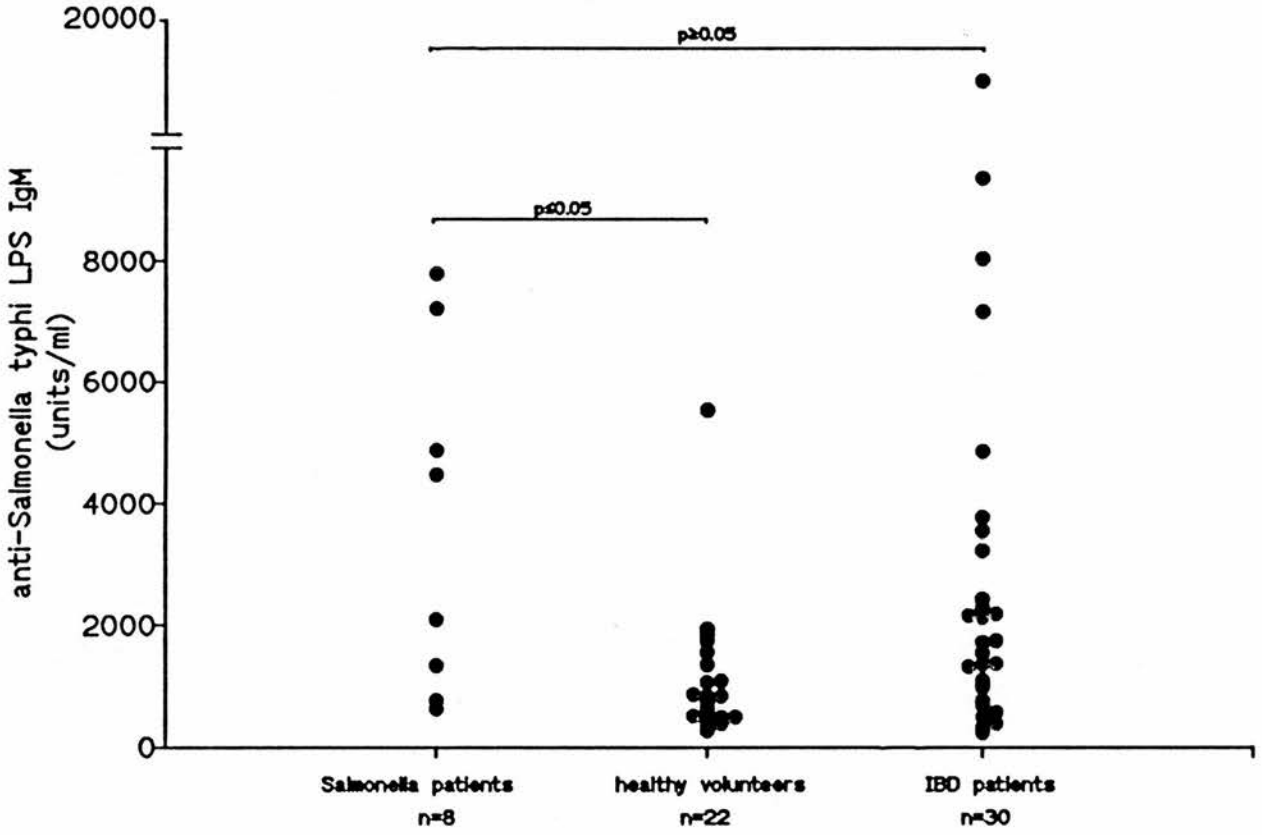


Fig. 7.VII. Concentrations of anti-*Salmonella typhi* LPS IgM antibodies in sera of patients who had had salmonella infection as compared to healthy volunteers and IBD patients.

Chapter VIII

EFFECT OF CIGARETTE SMOKING ON GUT IMMUNITY:

DOES SMOKING SUPPRESS INTESTINAL IMMUNE RESPONSES ?

Introduction

Cigarette smoking has been shown to modify the systemic immune responses in animals as well as in humans (see chapter 2). Both the cellular and the humoral aspects of the immune system have been shown to be affected by cigarette smoking.

In spite of the extensive research work into the influence of cigarette smoking on the systemic immune system, there have been few studies, *in vivo*, investigating the effect of smoking on the mucosal immune system. The discovery, in the last few years, of an epidemiological relationship between cigarette smoking and inflammatory bowel diseases (IBD) (see chapter 2) has markedly increased the interest in the immunological changes associated with cigarette smoking, and of particular interest is the effect on the intestinal mucosal immune system.

Barton and colleagues (1990) investigated the effect of cigarette smoking on the concentration of immunoglobulins in pure parotid saliva obtained from healthy smokers, non-smokers and ex-smokers and found that the concentration of salivary IgA in the smokers group was significantly reduced compared to the non-smokers control. The concentration of salivary IgM, on the other hand, was found to be significantly increased in the smokers group. In the above study, the decrease in the salivary IgA in the smokers group was found to be

dose-related and reversible after cessation of smoking. However, further studies on saliva showed that the immunological changes in the saliva do not necessarily represent the profile of the immune responses at the intestinal mucosa (O'Mahony et al, 1991).

Srivastava et al (1991) investigated the effect of smoking on the humoral immune responses of the gut using the WGL technique in healthy subjects and patients with ulcerative colitis including both smokers and non-smokers. This study found no difference between immune responses in the intestinal mucosa of smokers and non-smokers and they concluded that smoking does not suppress the intestinal immune system. However, smokers in the above study were not heavy smokers (smoked as little as 10 cigarettes per day) and the period of smoking was relatively short (one year).

Therefore, in this study the WGL technique was exploited to investigate further the influence of heavy smoking (20 cigarette per day or more) for a long period (at least 3 years). Alteration of the mucosal immunoglobulins and antigen specific antibodies may be important, not so much in their own right, but as markers of underlying alteration in the intestinal immune function. If further work confirms the presence of mucosal IgA deficiency among heavy smokers, the implications are considerable, not only for oral immunisation programmes, but also in relation to the aetiologies of smoking-related diseases.

Subjects and methods

The 22 adult healthy British volunteers involved in the study of the effect of the oral typhoid vaccine Ty21a on gut immunity described in chapter 6 were also recruited for this study. Volunteers were divided into 14 heavy smokers (one female and 13 males) and 8 non-smokers (one female and 7 males). The mean value of the age of the smokers group was 27.43 (± 5.00 SD) while the mean of the age of the non-smokers group was 32.75 (± 7.27 SD). The mean value of the number of cigarettes smoked by the smokers group was 21.43 (± 4.13 SD) per day for at least the past 3 years. All volunteers drank less than 20 units of alcohol per week.

Intestinal fluids (obtained by WGL technique) and venous blood samples were obtained from all volunteers before and 3 weeks after the administration of the oral typhoid vaccine Ty21a as described in chapter 6.

Assays performed

1. ELISA: for the quantitative measurement of total immunoglobulins in the WGL fluid before and after vaccination.

2. ELISA: for the quantitative measurement of anti-*Salmonella typhi* LPS antibodies in WGL fluids and serum before and after vaccination.

The concentrations of total IgA, IgM, and anti-*Salmonella typhi* LPS IgA, IgM and IgG in WGL fluid of the smokers group were compared with total immunoglobulins and antigen-specific antibodies of the non-smokers group before and 3 weeks after administration of the oral typhoid vaccine Ty21a.

Levels of serum total IgA, IgM, and IgG as well as anti-*Salmonella typhi* LPS IgA, IgM and IgG antibodies of the smokers and non-smokers groups obtained before and 3 weeks after vaccination were also studied comparing the concentration of those immunoglobulins and antigen-specific antibodies in the smokers and non-smokers groups.

Since the results obtained in this study were found to be non-parametric and independent, the Mann-Whitney U-test was used for the statistical analysis of the data.

Results

1. **The concentration of TIgA in WGL fluid of smokers and non-smokers before and after vaccination**: the median value of the pre-vaccination

TIgA of the smokers group was 71.05 μg/ml with a range of 10.00-173.20 while the median of the pre-vaccination TIgA of the non-smokers group was 83.80 μg/ml with a range of 24.00-110.40. The median value of the post-vaccination TIgA of the smokers group was 72.00 μg/ml with a range of 20.90-225.30 while the median of the post-vaccination TIgA of the non-smokers group was 57.70 μg/ml with a range of 28.20-106.20. There was no significant difference between the pre-vaccination TIgA of the smokers and non-smokers groups ($p=0.919$) (Fig. 8.I). Likewise, there was no significant difference between the post-vaccination TIgA of the smokers and non-smokers groups ($p=0.322$). (Fig. 8.I).

2. **The concentration of TIgM in WGL fluid of smokers and non-smokers before and after vaccination**: The median value of the pre-vaccination TIgM of the smokers group was 1.75 μg/ml with a range of 0.10-9.50 while the median of the pre-vaccination TIgM of the non-smokers group was 2.70 μg/ml with a range of 0.10-4.40. The median value of the post-vaccination TIgM of the smokers group was 1.60 μg/ml with a range of 0.10-5.10 while the post-vaccination TIgM of the non-smokers group was 1.90 μg/ml with a range of 0.50-4.30. There was no significant difference between the pre-vaccination TIgM of the smokers and non-smokers groups ($p=0.633$). (Fig. 8.II). There was also no significant difference between

the post-vaccination TIgM of smokers and non-smokers groups (p=0.609). (Fig. 8.II).

3. **Anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of smokers and non-smokers before and after vaccination**: the median value of the pre-vaccination anti-*Salmonella typhi* LPS IgA antibodies of the smokers group was 52.1 units/mg of TIgA with a range of 12.0-265.1 while the median of the pre-vaccination IgA antibodies of the non-smokers group was 88.2 units/mg of TIgA with a range of 15.3-230.0. The median value of the post-vaccination anti-*Salmonella typhi* LPS IgA antibodies of the smokers group was 91.8 units/mg of TIgA with a range of 6.9-205.7 while the median of the post-vaccination IgA antibodies of the non-smokers group was 132.4 units/mg of TIgA with a range of 18.0-1519.0. There was no significant difference between the pre-vaccination anti-*Salmonella typhi* LPS IgA antibodies of the smokers and non-smokers groups (p=0.290) (Fig. 8.III). There was also no significant difference between the post-vaccination IgA antibodies of the smokers and non-smokers groups (p=0.109) (Fig. 8.III).

4. **Changes in anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of smokers and non-smokers before and after vaccination**: studies described in chapter 6 have shown that there was an overall significant increase, after vaccination, in the titre of anti-*Salmonella typhi* LPS IgA

antibodies in WGL fluid of the volunteers involved in the study. However, when the changes in the anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of the smokers group was compared with the changes in IgA antibodies in WGL fluid of the non-smokers group, the difference was found to be not significant ($p=0.562$).

5. **Anti-*Salmonella typhi* LPS IgM antibodies in WGL fluid of smokers and non-smokers before and after vaccination**: there was no significant difference between anti-*Salmonella typhi* LPS IgM antibodies in WGL fluid of smokers and non-smokers before or after vaccination (p values were 0.065, and 0.075 respectively).

6. **Total IgA, IgM, and IgG in the serum of the smokers and non-smokers before and after vaccination**: there were no significant differences between total IgA, IgM, and IgG in the sera of the smokers and non-smokers groups before vaccination (p values were 0.172, 0.759, and 0.838 for IgA, IgM and IgG respectively) or after vaccination (p values were 0.136, 0.574 and 0.719 for IgA, IgM and IgG respectively).

7. **Anti-*Salmonella typhi* LPS IgA, IgM, and IgG antibodies in serum of smokers and non-smokers before and after vaccination**: the median values of the pre-vaccination anti-*Salmonella typhi* LPS IgA, IgM, and IgG antibodies in the serum of the smokers were 516.0, 790.5, and 524.5

units/ml respectively (ranges were 46.0-1438.0, 386.0-5540.0 and 167.0-1403.0 respectively) while the median values of the pre-vaccination IgA, IgM, and IgG antibodies in the serum of the non-smokers were 263.9, 740.2, and 460.8 units/ml respectively (ranges were 116.0-7150.0, 277.0-1938.0 and 262.0-4499.0 respectively). The median value of the post-vaccination anti-*Salmonella typhi* LPS IgA, IgM, and IgG antibodies in the serum of the smokers were 674.9, 773.5 and 665.8 units/ml respectively (ranges were 42.0-3375.0, 373.0-6446.0 and 169.0-5265.0 respectively) while the median of the these antibodies in the serum of the non-smokers were 1250.4, 1183.0, and 1170.7 units/ml respectively (ranges were 283.0-7274.0, 460.0-3367.0 and 240.0-46267.0). There were no significant differences between the pre-vaccination anti-*Salmonella typhi* LPS IgA, IgM, and IgG antibodies in the serum of the smokers and non-smokers groups (p values were 0.707, 0.657 and 0.759 respectively) (Fig. 8.IV). Likewise, there were no significant differences between the post-vaccination IgA, IgM, and IgG antibodies in the sera of the smokers and non-smokers group (p value were 0.232, 0.394 and 0.517 respectively) (Fig. 8.IV).

Conclusion

The above results indicated that there were no significant differences between the healthy heavy smokers and non-smokers volunteers in terms of intestinal as

well as systemic immunoglobulins and antigen-specific antibodies. In spite of the fact that administration of an intestinal mucosal immunogen such as the oral live typhoid vaccine Ty21a has been shown to produce an overall significant increase in the intestinal (as well as systemic) antigen-specific antibodies of the healthy volunteers (see chapter 6), yet the differences between the changes in the smokers and non-smokers groups were found to be not significant.

The above findings suggest that smoking does not significantly suppress the intestinal mucosal immunoglobulins nor the antigen-specific antibodies of the healthy volunteers when challenged with live antigen. Therefore, the protective effect of smoking, if any, in patients with ulcerative colitis may be due some other mechanism(s) that has (have) not yet been well-understood.

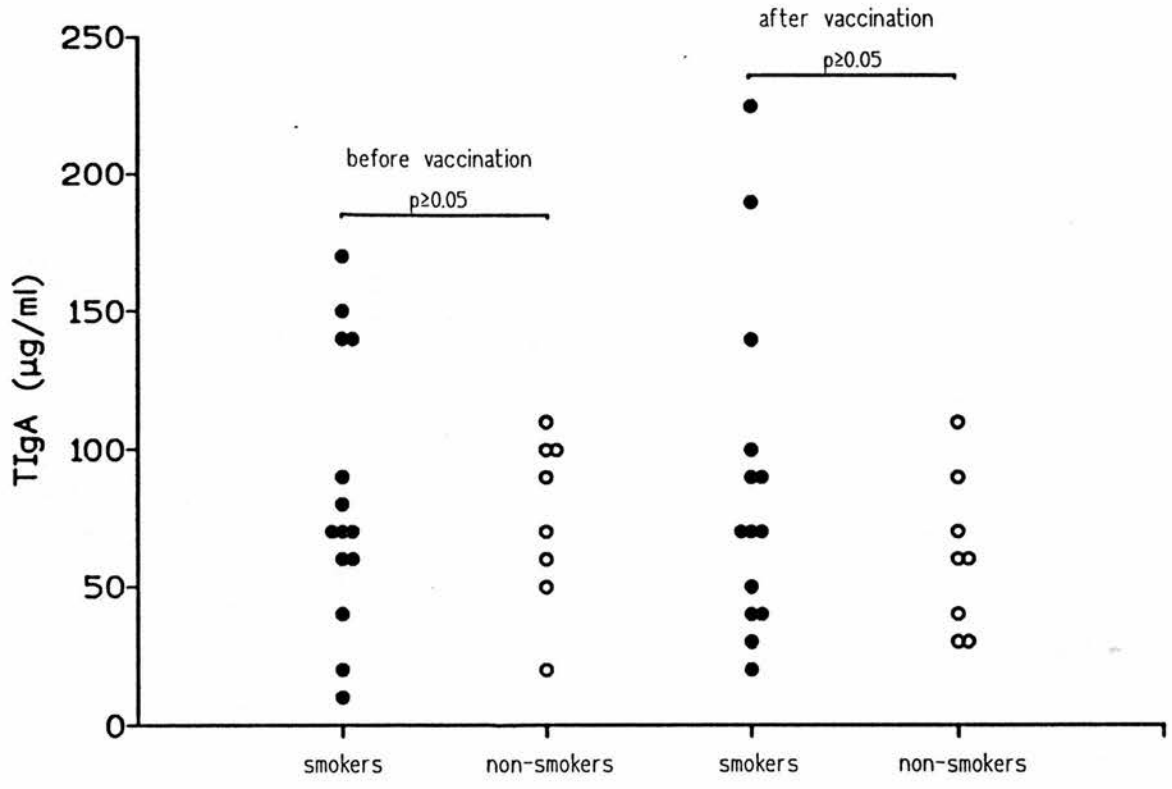


Fig. 8.I. Concentrations of TIgA in WGL fluid of healthy smokers (n=14) and non-smokers (n=8) before and after vaccination with the oral typhoid vaccine Ty21a.

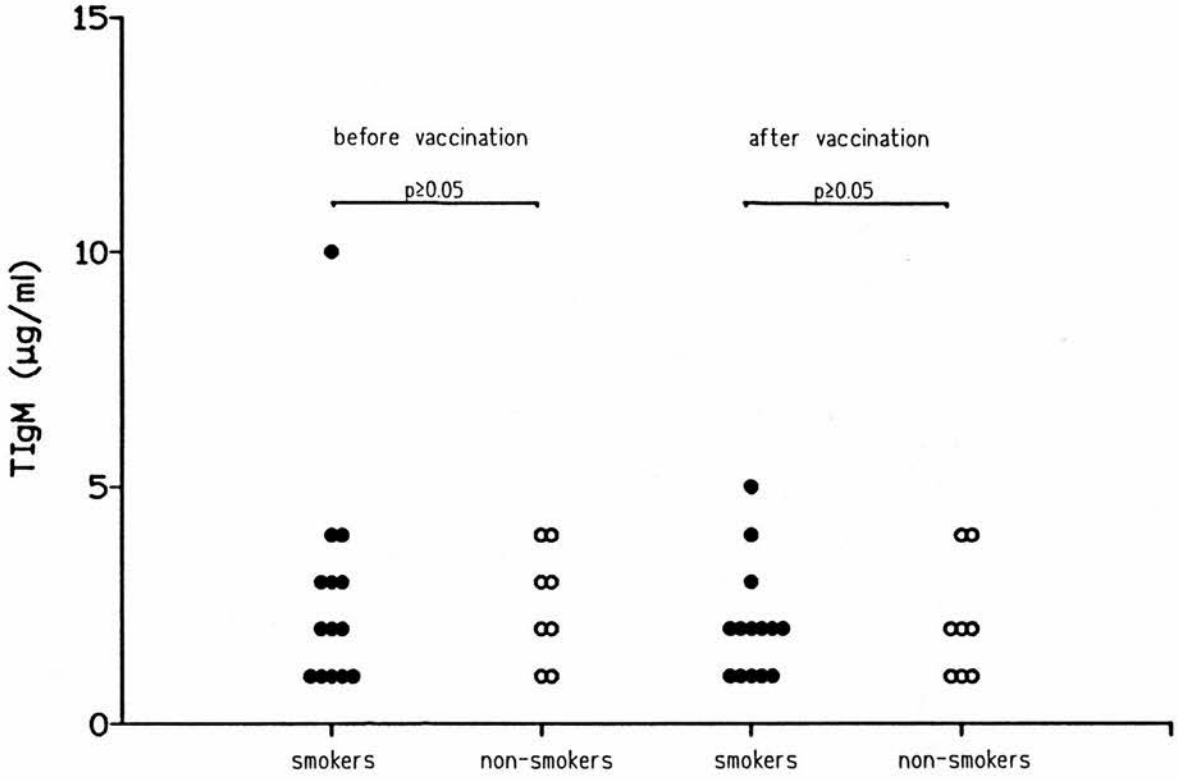


Fig. 8.II. Concentrations of TIgM in WGL fluid of healthy smokers (n=14) and non-smokers (n=8) before and after vaccination with the oral typhoid vaccine Ty21a.

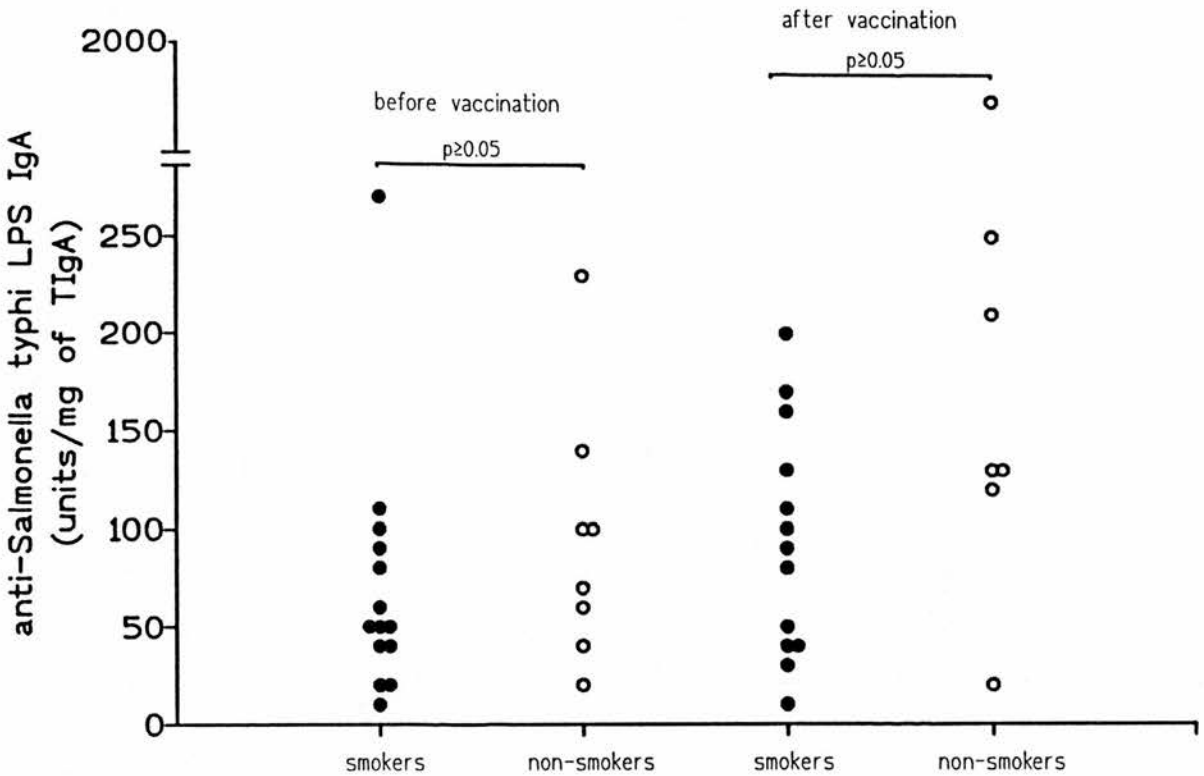


Fig. 8.III. Concentrations of anti-*S. typhi* LPS IgA antibodies in WGL fluid of healthy smokers (n=14) and non-smokers (n=8) before and after vaccination with the oral typhoid vaccine Ty21a.

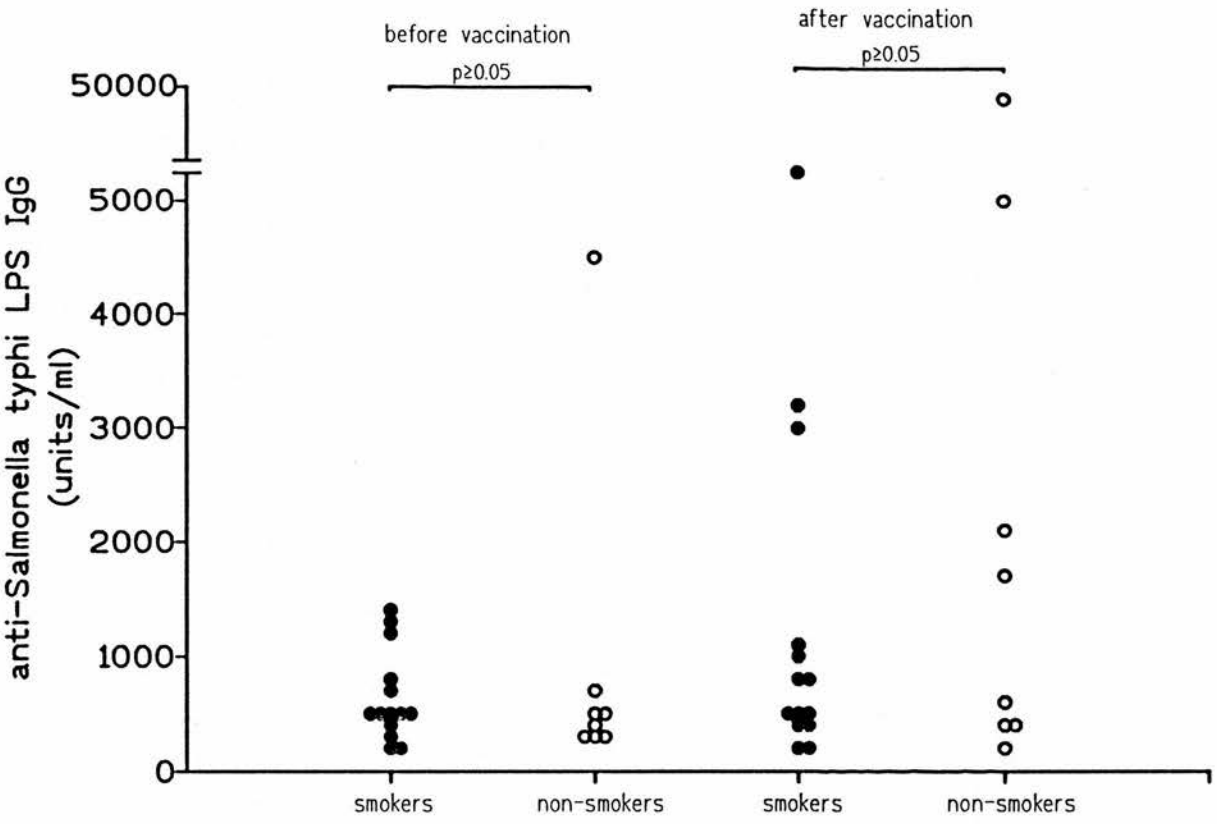


Fig. 8.IV. Concentrations of anti-*S. typhi* LPS IgG antibodies in sera of healthy smokers (n=14) and non-smokers (n=8) before and after vaccination with the oral typhoid vaccine Ty21a.

Chapter IX

INTESTINAL MUCOSAL IgA DEFICIENCY

Introduction

Normally, IgA is found in almost all body fluids. The overall production of IgA was estimated as 66 mg/kg body weight per day compared to 34, 7.9, 0.4, 0.02 mg/kg body weight per day for IgG, IgM, IgD and IgE respectively (Mestecky and McGhee, 1987; Hanson and Brandtzaeg, 1988). In serum, the level of IgA, with a mean value of 200±61 mg/dl, is ranked as second after the IgG level which has a mean value of 1158±31 mg/dl (Conley and Delacroix, 1987), while on the mucosal surface IgA is by far the predominant immunoglobulin with the daily amount of secretory IgA (SIgA) secreted into the gut lumen estimated as 40 mg/kg body weight (i.e. more than the total daily production of IgG) (Conley and Delacroix, 1987). Levels of IgA in serum do not represent the overall rate of production of IgA simply because approximately 2/3 of IgA is locally produced in the mucosa and secreted into the external secretions and never enters the systemic pool. In addition, the half-life of IgA was found to be relatively short (estimated as 3-6 days compared to the half-life of 20-23 days of IgG) (Mestecky et al, 1991).

In serum, IgA is present mainly as the 7S monomeric form and is thought to be produced by bone marrow plasma cells while IgA on the mucosal surfaces is produced by local plasma cells and secreted onto the mucosal surfaces mainly as the 11S dimeric form. The systemic and mucosal compartments have been shown to be separate with little contribution, if any, of serum IgA to the mucosal secretion (Conley and Delacroix, 1987; Mestecky, 1988; O'Mahony et al, 1991).

In humans, the catabolic site(s) of IgA is/are not, as yet, known. However, an Fcα receptor (FcαR) has been recently identified on phagocytic white blood cells (neutrophils and macrophages) and has been suggested to be involved in the catabolism of IgA antibodies as well as clearance of IgA immune complexes from serum (Schaffer et al, 1993). The structure and function of IgA were described in chapter 2.

IgA deficiency

Systemic IgA deficiency. Classification of primary immunodeficiency diseases by the World Health Organisation (WHO), in 1983, described at least 13 disorders characterised predominantly by antibody deficiency (Buckley, 1986). Selective IgA deficiency, i.e. 50 μg/ml or less of IgA in serum (Hong and Ammann, 1989), is regarded as the most common type of primary immunodeficiency with a rate of incidence of 1 in 600 (Hanson et al, 1983), predominantly among Caucasians and more commonly in males than in females (Buckley, 1986). This type of antibody deficiency has been found in asymptomatic individuals as well as patients with a variety of infections including gastrointestinal, respiratory, and urinary tract infections (West et al, 1962).

Selective IgA deficiency may also be acquired as a complication of certain drugs. Administration of phenytoin, a drug used for the treatment of epileptic seizures,

has been reported to cause IgA deficiency in 20-40% of patients treated with it (Sorrell et al, 1971; Aarli, 1976). Other drugs such as sulphasalazine, used for the treatment of rheumatoid arthritis and inflammatory bowel disease (Delamere et al, 1983), antimalarial agents (Schaffer et al, 1993) and captopril, used for the treatment of hypertension and congestive heart failure (Hammarstrong et al, 1991) have also been reported to be involved in the acquisition of IgA deficiency.

Environmental factors such as congenital and neonatal infections have been implicated in the acquisition of IgA deficiency. Congenital infection with cytomegalovirus and *toxoplasma gondii* were reported to be associated with subsequent IgA deficiency (Rosen, 1980). Intrauterine infection with rubella virus was also reported to be associated with subsequent IgA deficiency (Soothill et al, 1966). Healthy individuals may also have transient IgA deficiency following infection with Epistein-Barr virus (EBV) (Saulsbury, 1989).

The association with IgA deficiency of ataxia telangiectasia, an autosomal recessive syndrome that is characterised by progressive ataxia and occulo-cutaneous telangiectasia, autoimmune and atopic diseases (Swift, 1990) has attracted attention to the possibility that a genetic factor may be the cause of IgA deficiency. A genetic factor may cause a defect in the expression of certain regulatory mediators which are important for maturation and differentiation of IgA-producing cells. IgA deficiency may be associated with certain human leukocyte antigens (HLA) which encode genes that have an immunoregulatory function particularly those genes

involved in antigen presentation and in the interaction between T cells and B cells (Schaffer et al, 1993). Of particular interest, are antigens of the HLA-A and -B classes such as HLA-A1, -A2 and -B8 antigens (Ambrus et al, 1977). Whether the presence of these HLA antigens is a predisposing factor to both IgA deficiency as well as to the underlying diseases or is primarily associated with the underlying diseases rather than IgA deficiency, is not, as yet, clear.

Selective IgA deficiency has been reported to be associated with a marked increase in IgM-producing cells and, to a lesser extent, IgG-producing cells in the lamina propria of the intestinal mucosa (Brandtzaeg et al, 1968; Savilhati, 1973). The increase in the number of IgM-producing cells in the lamina propria of subjects with IgA deficiency was found to be associated with an increase in the local production of secretory IgM (SIgM) in saliva (Brandtzaeg et al, 1968) and intestinal secretions (Haneberg and Aarskog, 1975) probably as a compensatory mechanism. Compensatory SIgM may act as a protective antibody on the mucosal surfaces of individuals with IgA deficiency and this may account for the lack of gastrointestinal symptoms in some subjects with IgA deficiency (Arnold et al, 1977). However, the binding of IgM to the secretory component (SC) was found to be less stable than the binding of IgA (Brandtzaeg, 1975) rendering SIgM less effective than SIgA in protection of mucosal surfaces of the gut.

The clinical implications of IgA deficiency are markedly variable: many subjects with IgA deficiency are otherwise healthy. However, patients with IgA deficiency

have been reported to have a variety of infections such as recurrent respiratory infections (South et al, 1965; Burks and Steel, 1986), chronic gastrointestinal infections (Schaffer et al, 1993) viral hepatitis (Ammann and Hong, 1971), septicaemia (Klemola, 1987) and meningoencephalitis (Cursons et al, 1979).

Allergic disorders such as allergic conjunctivitis, rhinitis, urticaria, atopic eczema and bronchial asthma have been reported to be associated with IgA deficiency (Ostermaard, 1980; Burks and Steel, 1986). Rheumatoid arthritis and systemic lupus erythematosus (SLE) were also reported to occur in patients with IgA deficiency. The latter disease was reported to occur in 5-7% of subjects with IgA deficiency (Cassidy et al, 1969).

A variety of gastrointestinal disorders such as coeliac disease, pancreatic insufficiency (Penny et al, 1971), regional enteritis and ulcerative colitis (Ammann and Hong, 1971), have been found to be associated with IgA deficiency. Primary biliary cirrhosis and chronic active hepatitis are hepatic disorders reported to be associated with IgA deficiency (James et al, 1986; Klemola, 1987).

Subjects with IgA deficiency have been reported to have high levels of IgG antibodies against cow's milk and serum proteins (Buckley, 1986).

Skin disorders that may be found in subjects with IgA deficiency include pyoderma gangrenosum, vitiligo (Wolf and Wolf, 1982), haemorrhagic purpura (Bundino and Zina, 1984) and Henoch-Schoenlein syndrome (Martini et al, 1985).

Mucosal IgA deficiency. The statement "IgA deficiency" has always referred to IgA deficiency in serum and little, if anything, is known about mucosal IgA deficiency. Patients with selective IgA deficiency usually exhibit the absence or near absence of IgA in both serum and mucosal surfaces. Therefore, it was an interesting observation to find, during research into gut immunity, that there were some subjects who had very low levels or absence of IgA in their WGL fluid but had normal levels of IgA in their serum.

In one case report (Strober et al, 1976), the lack of IgA in the intestinal fluid in a 15-year old boy who had very low level of intestinal (jejunal) IgA (but had a normal level of IgA in serum) was attributed to the lack of the SC which prevented the normal secretion of locally produced IgA into the intestinal lumen.

Apart from the above case report, the phenomenon of normal levels of IgA in serum but low levels or absence of IgA in the intestinal secretions has not been investigated before. Such investigation may provide instructive insights into the physiology of IgA production and may result in a better understanding of the immunoregulatory mechanisms behind this disorder.

Aim of the study

The discovery of subjects, during my research into gastrointestinal immunity, who had normal levels of IgA in serum but had low levels or absence of IgA in their WGL fluid was a chance finding of a previously unrecognised form of humoral immunodeficiency. Therefore, it was important to investigate this phenomenon in healthy subjects and patients who had low levels or absence of IgA in their intestinal fluids (WGL fluid or jejunal aspirate) highlighting subjects who had normal levels of IgA in serum. Counting of IgA-containing cells in the lamina propria of those subjects was carried out in their duodenal or jejunal biopsies.

Patients and methods

Specimens of WGL fluid obtained from healthy individuals and patients with a variety of gastrointestinal diseases during 1991 and 1992 were screened looking for specimens with very low levels or absence of IgA. A total of 391 WGL specimens were assayed and the concentrations of TIgA in these specimens were measured. Specimens containing concentrations of <10 μg/ml of total IgA (TIgA) in WGL fluid were regarded as IgA deficient. This is because analysis of the concentration of TIgA in WGL fluid of healthy volunteers, patients with IBD or salmonella infection (chapters 5, 6 and 7) has shown that a concentration of TIgA of <10 μg/ml is a very low level compared to the levels obtained from the above

mentioned groups (Table 9.IV). Specimens of WGL fluid obtained during 1991 and 1992 were collected and processed as described in chapter 3 and stored at -70 °C.

Jejunal aspirates collected (by Sister Crichton, G.I Annex using Crosby's capsule) during 1991 and 1992 from patients with a variety of gastrointestinal diseases were screened looking for IgA deficient specimens. A total of 238 specimens of jejunal aspirates were assayed (by Dr. Arranz, a research fellow and Mr. Anderson, a technician in the G.I laboratory). Specimens containing < 10 μg/ml of TIgA were regarded as IgA deficient. Analysis of the concentration of TIgA in jejunal aspirates in normal controls and patients with treated or untreated coeliac disease (Arranz, 1993) has shown that a concentration of TIgA in jejunal aspirate of < 10 g/ml is a low value compared to the levels of TIgA in other groups (Table 9.V).

ELISA was used for the quantitative measurements of TIgA and TIgM in WGL fluid and jejunal aspirates. The concentrations of IgA, IgM and IgG in serum collected at the same time as the WGL fluids or jejunal aspirates, of those subjects who were found to have intestinal IgA deficiency were measured by the Department of Clinical Biochemistry.

The number of IgA-containing plasma cells was counted in the lamina propria of 4 duodenal biopsies and one colonic biopsy obtained from 5 cases found to have zero or very low levels of TIgA in their WGL fluids but normal levels of IgA in serum.

No relevant biopsy was available for one case found to have no TIgA in WGL fluid but a normal level of IgA in serum. A set of slides containing 11 jejunal biopsies from 11 patients who had very low levels or absent TIgA in jejunal aspirates and 3 biopsies from 3 normal subjects who had normal levels of TIgA in jejunal aspirates and in serum (TIgA in jejunal aspirates ranged between 70.2 and 178.7 μg/ml and in serum between 1.91 and 2.03 mg/ml) was mixed and screened blindly looking for IgA-containing cells in the lamina propria after assigning a special code for every slide. Slides containing the duodenal or jejunal biopsies were stained immunohistochemically (by Mr. Bode, a MLSO in the G.I laboratory) and cell counting was carried out using an image analyser as described in chapter 3. In every slide, about 50 fields were examined systematically in horizontal and vertical directions under the x100 objective lens.

IgA-containing plasma cells in duodenal biopsies of 10 young subjects (aged <50 years) were counted (by Dr. Bruce, a research fellow) using the image analyser and the range of the numbers obtained was used as a normal control. These subjects presented to the out-patient clinic with a variety of symptoms but investigation revealed no abnormality and they were found to be immunologically normal.

Results

Measurement of the concentrations of TIgA and TIgM in the specimens of WGL fluid of the normal individuals and patients with a variety of gastrointestinal diseases collected during 1991 and 1992 revealed the presence of 7 patients with levels of TIgA ranged between zero and 5.2 μg/ml (table 9.I) and levels of TIgM ranged between zero and 7.1 (within normal limits for IgM). Apart from one subject, who had pan-hypogammaglobulinaemia, the concentrations of IgA, IgM and IgG in the sera of the above 7 subjects were found to be within the normal ranges and ranged between 2.01 and 4.14, 0.50 and 3.10 and between 8.00 and 21.83 mg/ml for IgA, IgM and IgG respectively.

Measurement of the concentrations of TIgA and TIgM in the jejunal aspirates of normal individuals and patients with a variety of gastrointestinal diseases collected during 1991 and 1992 revealed the presence of 15 patients with levels of TIgA ranged between zero and 9.7 μg/ml (table 9.II) and levels of TIgM ranged between zero and 10 μg/ml. Apart from one subject who had selective IgA deficiency, the concentrations of IgA, IgM and IgG in the sera of those subjects who had very low levels or absent IgA in their jejunal aspirates were found to be within the normal range and ranged between 1.49 and 3.41, 0.40 and 3.00 and between 7.74 and 16.18 mg/ml for IgA, IgM and IgG respectively.

In the group of subjects who had very low levels or absence of IgA in their WGL fluids, counting of IgA-containing plasma cells in the lamina propria of a duodenal biopsy of one subject revealed the presence of 9 IgA-containing plasma cells/mm^2 lamina propria while counting of IgA-containing plasma cells in the lamina propria of a duodenal biopsy of another subject revealed the presence of 19 cells/mm^2 lamina propria (Table 9.I).

In the group of subjects who had very low levels or absence of IgA in their jejunal aspirates, counting of IgA-containing plasma cells in the lamina propria of 11 jejunal biopsies revealed the presence of numbers of IgA-containing plasma cells ranged between zero and 900 cells/mm^2 lamina propria with 3 subjects having no IgA-containing plasma cells in the lamina propria (Table 9.II). Counting of IgA-containing plasma cells in the lamina propria of the three subjects used as a normal control revealed the presence of numbers of IgA-containing cells ranged between 17 and 423 cell/mm^2 lamina propria (Table 9.III) (Fig. 9.I).

The three subjects who had low levels or absent TIgA in jejunal aspirates (2 had normal levels of IgA in serum but the third subject had selective IgA deficiency) and had no IgA-containing plasma cells in the lamina propria had presented with abdominal pain accompanied by loose stools or diarrhoea. Apart from one case who had selective IgA deficiency (and a history of non-insulin dependent diabetes mellitus), clinical investigation revealed no abnormality.

Counting of IgA-containing plasma cells in the duodenal biopsies of 10 subjects, used as a normal controls, showed the presence of numbers ranged between 255 and 948 cells/mm^2 lamina propria.

Conclusion

Studies on WGL fluid of normal individuals and patients with a variety of gastrointestinal diseases collected during 1991 and 1992 revealed the presence of subjects with absent or near absent levels of IgA in their WGL fluids but with normal levels of IgA in serum. Counting of IgA-containing plasma cells in the lamina propria of 2 duodenal biopsies from 2 subjects who had zero levels of TIgA in their WGL fluid revealed the presence of a very small number of cells/mm^2 lamina propria. The above results suggested the presence of a previously unrecognised form of IgA immunodeficiency i.e. intestinal IgA deficiency, the implication of which is, as yet, unknown.

Screening of jejunal aspirates of patients and normal individuals collected during 1991 and 1992 revealed the presence of subjects with very low levels or absence of IgA in their jejunal aspirates but normal levels of IgA in serum. Counting IgA-containing plasma cells in the lamina propria of jejunal biopsies from these subjects revealed the presence of 3 subjects who had no IgA-containing cells. However, counting of IgA-containing plasma cells in the lamina propria of jejunal biopsies

from other subjects who had absent or near absent levels of TIgA in jejunal aspirates showed that some biopsies contained a number of IgA-containing plasma cells that overlapped with the numbers of IgA-containing plasma cells in the lamina propria of normal controls with normal levels of TIgA in jejunal aspirates. Furthermore, one subject (control 3, table 9.III) had a small number of IgA-containing plasma cells/mm^2 lamina propria although he had normal level of TIgA in his jejunal aspirate.

These results indicated that the jejunal aspirates used in this study were not reliable material in which to study immunoglobulin concentrations in the gut. Jejunal aspirate collection was not one of the protocols of my study: a scientist in the G.I laboratory has reported that the technical steps by which jejunal aspirates were collected may result in variable but marked dilution of the jejunal fluid during the process of collection, resulting in false low values or absence of IgA measured in some of the samples collected by the above method. This was due to a technical error involving flushing of Crosby's capsule tubes with normal saline just before obtaining jejunal biopsies (to clear the intubation system from any mucous or other tissue debris). Thereafter, when jejunal aspirate was collected, the remnants of the flushing fluid were collected with the jejunal fluid resulting in its dilution.

Whether the unexplained abdominal pain and idiopathic diarrhoea found in three subjects found to have low levels or absent TIgA in jejunal aspirates and had no

IgA-containing plasma cells in the lamina propria is related to the intestinal IgA deficiency or not, is not, as yet, clear.

subject	age	sex	diagnosis	date of lavage	TIgA(WGL) μg/ml	IgA(serum) mg/ml	type of biopsy	date of biopsy	IgA cells
1	57	M	idiopathic diarrhoea	06/05/91	none	2.64	duodenal	28/02/91	9
2	55	F	idiopathic diarrhoea	01/07/91	none	2.35	duodenal	14/06/91	19
3	27	M	Crohn's disease	04/02/91	5.2	2.01	jejunal	11/02/91	333
4	91	F	Crohn's disease	22/10/92	none	3.16	NA	-	-
5	40	M	hypogammaglobulinaemia	09/10/91	none	0.14	duodenal	10/10/91	nil
6	69	F	collagenous colitis	04/11/91	none	4.14	duodenal	03/11/91	414
7	58	F	rectal cancer	01/05/92	none	2.37	colonic	21/01/92	304

Table 9.I. Results of screening of whole gut lavage (WGL) fluid specimens collected during 1991 and 1992 (391 specimens) looking for IgA deficient specimens. NA=biopsy were not available. IgA cells=IgA-containing plasma cells/mm^2 lamina propria.

subject	age	sex	diagnosis	TIgA (JA) µg/ml	IgA (serum) mg/ml	type of biopsy	IgA cells
1	60	F	selective IgA deficiency, DM	5.2	0.14	jejunal	none
2	36	M	IBS	7.4	2.10	jejunal	702
3	47	F	IBS	8.8	2.30	jejunal	270
4	24	F	idiopathic diarrhoea	4.0	1.55	jejunal	900
5	30	M	idiopathic diarrhoea	4.9	1.49	jejunal	none
6	58	M	idiopathic diarrhoea	none	1.54	jejunal	none
7	24	F	idiopathic diarrhoea	7.7	2.59	jejunal	467
8	47	F	coeliac disease	9.7	1.69	ND	-
9	16	F	coeliac disease	8.6	1.42	jejunal	562
10	36	M	coeliac disease	0.2	1.13	ND	-
11	57	M	coeliac disease	4.8	3.41	ND	-
12	58	M	ulcerative colitis	5.4	2.97	ND	-
13	65	F	Crohn's disease	8.8	2.30	jejunal	234
14	65	M	colonisation	7.3	3.01	jejunal	114
15	25	M	DM, IBS	3.4	1.07	jejunal	281

Table 9.II. Results of screening of jejunal aspirate (JA) specimens collected during 1991 and 1992 looking for IgA deficient specimens. JA=jejunal aspirate, DM=diabetes mellitus. IBS=irritable bowel syndrome. ND=not done

subject	age	sex	diagnosis	TIgA (JA) μg/ml	IgA (serum) mg/ml	type of biopsy	IgA cells
1	18	F	unexplained abdominal pain	70.2	1.91	jejunal	423
2	40	F	ileo-rectal anastomosis	68.0	1.99	jejunal	303
3	41	M	iron deficiency anaemia	178.7	2.03	jejunal	17

Table 9.III. IgA in jejunal aspirates (JA) and in serum of three subjects used as a normal control in IgA deficiency in JA. IgA-containing plasma cell/mm^2 lamina propria are also shown in the table.

group	TIgA in WGLF (μg/ml)	TIgM in WGLF (μg/ml)	IgA in serum (mg/ml)
intestinal IgA deficient subjects (n=7)	nil-5.2	nil-7.1	2.01-4.14
healthy volunteers (n=22)	10-173.2	nil-9.5	1.60-5.50
IBD patients (n=59)	31-520	1.1-80.9	1.17-6.70
salmonella-infected patients (n=8)	24.5-520.6	2.3-16.8	1.20-4.17

Table 9.IV. Ranges of TIgA and TIgM in whole gut lavage fluid (WGLF) and IgA in serum of intestinal IgA deficient subjects as compared to healthy volunteers, patients with inflammatory bowel disease (IBD) and patients who had had naturally acquired salmonella infection.

group	TIgA in JA (μg/ml)	TIgM in JA (μg/ml	IgA in serum (mg/ml)
intestinal IgA deficient subjects (n=15)	0-9.7	0-10	1.07-3.41
normal control (n=25)	23-540.6	0-39.5	0.9-4.1
untreated coeliac disease patients (n=26)	25-2285	1.9-358	1.3-3.8
treated coeliac disease patients (n=15)	20-1558	0-174	0.7-3.3

Table 9.V. Ranges of TIgA and TIgM in jejunal aspirates (JA) and IgA in serum of IgA deficient subjects as compared to other groups (data of the normal control, untreated coeliac and treated coeliac patients were assayed by Dr. Arranz, G.I laboratory).

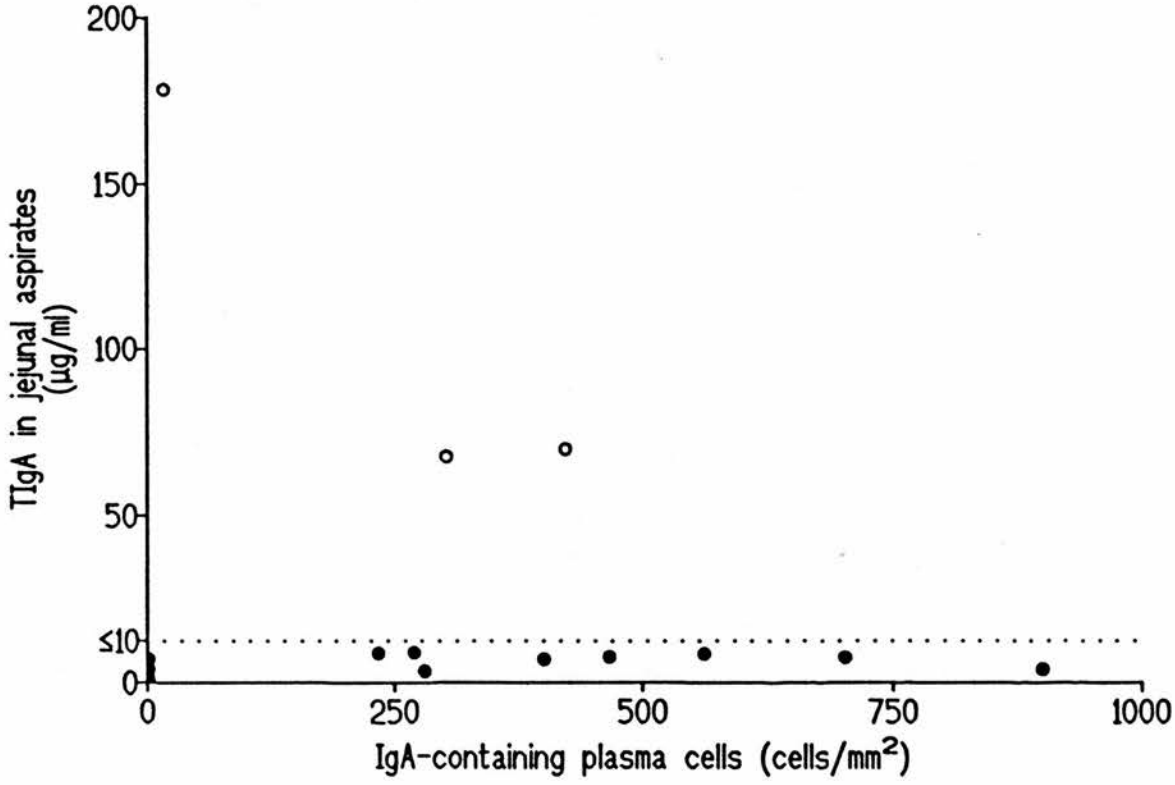


Fig. 9.I. Scatterogram showing the relationship between the IgA concentration and the IgA-containing plasma cells in jejunal biopsies of subjects with low levels of IgA in jejunal aspirate (n=11) and normal controls (n=3). •=subjects with low IgA concentrations in jejunal aspirate. o=normal controls.

Chapter X

GENERAL DISCUSSION AND CONCLUSION

Section 1

SERIAL LAVAGE STUDY

Establishing WGL fluid as a perfusate was important to validate its use in the research work described in this thesis as well as in other areas of clinical research into gut immunity. O'Mahony et al (1990) showed that once WGL specimens became clear there were no significant differences between levels of TIgA in two sequential lavage specimens. However, the analysis of a single parameter in just two sequential lavage specimens may not be sufficient to establish WGL fluid as a perfusate. Therefore, in the serial lavage study (described in chapter 4), 5 or 6 specimens were taken from every participant. The results showed that once the effluent fluid became clear there were no significant differences between levels of TIgA in the 5 or 6 serial lavage specimens collected over a period of about 1½ to 2 hours. In addition, levels of TIgM, albumin, α-1-antitrypsin, protease enzymes activity and anti-*Salmonella typhi* LPS antibodies were studied in 5 or 6 clear sequential lavage specimens and it was found that there were no significant differences between the first clear specimen and the succeeding 4 or 5 serial lavage specimens.

Regarding TIgG, there were no significant differences between sample number 1 and samples number 2 or 3 but there were significant differences between samples number 1 and samples number 4 and 5 of WGL fluid. Likewise, there were significant differences between levels of total proteins in sample number 1 and

sample number 5 of the sequential lavage specimens. This variation may be because WGL may actually be reducing gastrointestinal protein loss e.g. by removing an agent which damages or changes the permeability of the intestinal epithelium. However, inspection of the actual data makes the above explanation unlikely. This important issue is now being investigated further in the G.I laboratory, using the protocol I developed, in a group of patients with active IBD. Another explanation for the variations in the concentrations of TIgG and total proteins reported in the serial lavage study is that these are chance statistical findings in view of the many statistical comparisons made.

WGL fluid is now being used in the G.I. laboratory as material in which to study the cellular contents (such as neutrophils) and cytokines (such as IL-1, IL-6 and IL-8) of the intestinal secretions. However, the cellular and interleukin contents of WGL fluid in serial lavage specimens have not been studied and whether the cellular and cytokine contents are steady in the gut perfusate or not, is to be further investigated.

In another study, investigating the effects of the oral typhoid vaccine Ty21a on gut immunity of 22 healthy British volunteers (see chapter 6), the concentration of TIgA and TIgM in WGL fluid of the volunteers were measured before and 3 weeks after administering the vaccine. There were no significant differences between levels of TIgA or TIgM in WGL fluid of the volunteers before and after vaccination. The median value of the concentrations of TIgA in WGL fluid of the

22 healthy volunteers was found to be 73.25 μg/ml. The similarity in the concentrations of TIgA in WGL fluid collected before and 3 weeks after vaccination support the hypothesis that, when a standard and nurse-supervised protocol is used, WGL fluid is a perfusate and not just a bolus of material obtained during the process of WGL.

Several investigators have studied intestinal immunity by obtaining intestinal secretions (duodenal or jejunal aspirates) through intestinal intubation (see chapter 2). In those studies, the concentration of TIgA in intestinal secretions was estimated as 40 mg/kg body weight per day (Conley and Delacroix, 1987). In the technique of WGL the gut is perfused at a rate of 20 ml/minute. Therefore the daily production of TIgA, in an adult of 70 kg body weight, can be calculated as follows: TIgA in WGL fluid (μg/ml) X 20 X 60 X 24. The mean value of the rate of daily production of TIgA was found to be approximately 2.11 g/day. This value is close to the generally accepted value of the rate of daily production of TIgA of 2.8 g/day based on intestinal secretion of 40 mg/kg body weight/day.

Recently, a new technique for segmental jejunal (Knutson et al, 1989) or rectal and colonic (Raab et al, 1992) perfusion at a rate of 3 ml/minute has been described to study the quantitative release of soluble substances and cells in the perfusate. The volumes of perfusate recovered were reported to be equal to the infused volumes. Although the rate of production of TIgA in the above studies was not reported, the concentration of albumin in the jejunal perfusate was estimated as 31 μg/ml i.e.

0.134 g/day. This value is close to the mean of the concentrations of albumin in WGL fluid obtained from the healthy volunteers of 0.144 g/day. The technique of segmental perfusion of the gut is an invasive, inconvenient and demanding procedure that requires endoscopy and manipulative skills to position the tube in the target segment. Nevertheless, the above technique showed that a steady state of perfusion of a segment of the gut can be obtained.

Drinking the lavage solution at a standard rate seems to be important for obtaining optimal concentrations of intestinal immunoglobulins and antibodies in WGL fluid. A study in the G.I laboratory (unpublished work) has shown that drinking the lavage solution at 3 different rates produced 3 different concentrations of TIgA. Just as in the studies described in this thesis, concentrations of TIgA were similar in the sequentially collected WGL fluid specimens for any given session. In the above study, 3 healthy volunteers drank the lavage solution at rates of 1 litre/75 minutes, 1 litre/50 minutes and 1 litre/25 minutes each in 3 different sessions. It has been found that the shorter the period of drinking the same amount of the lavage solution the more diluted became the concentrations of TIgA. The above study emphasises the importance of standardisation of the rate of drinking the lavage solution and further supports the hypothesis of WGL fluid as a perfusate.

The use of the concentration of TIgG in WGL fluid as an index of disease activity in inflammatory bowel disease is an important application of the use of WGL fluid in clinical tests. Another potential application for WGL is investigating occult

blood loss from the gut of patients with a variety of gastrointestinal diseases by measuring the haemoglobin contents of WGL fluid. However, none of the 8 cases from whom serial lavage specimens were found to be technically suitable to be included in the study was frankly bleeding during the serial lavage study. Therefore, further study is required to confirm that blood content of WGL fluid is steady in serial lavage specimens.

In a review of the WGL technique performed in the G.I. Unit during a period of 18 months in 252 patients and 24 healthy volunteers (personal communication, Professor Ferguson, University of Edinburgh) the success rate, defined as passage of clear fluid per rectum, achieved was found to be 96%. The high success rate may be due to the fact that the procedure of WGL was supervised by experienced nurses who ensured accurate recording of the fluid intake and output as well as encouraging subjects to continue drinking till clear fluid passed per rectum.

Results of 3 out of 11 cases recruited for the serial lavage study were excluded from the final analysis of the data. An eighty two-year old lady (patient number 5, table 1) was excluded because at least 2 specimens of WGL fluid from this lady were found to be contaminated with urine. A twenty three-year old lady who had a diagnosis of ulcerative colitis (patient number 10, table 1) was excluded because she had excessive bleeding per rectum during the procedure of WGL resulting in high blood contents in WGL specimens. Results obtained from patient number 4 (table 1) were excluded because this patient failed to continue the experiment after

passing 4 small volumes clear lavage fluid. However, apart from TIgG in the WGL fluid of patient number 5, the raw data as well as the graphical representation of the results obtained from the above mentioned excluded cases were found to be consistent with those results obtained from the 8 cases included in the study.

The 3 excluded cases highlight difficulties which may be encountered during the procedure of WGL and illustrate situations where results should be carefully interpreted. For instance, specimens from very old subjects or from bleeding patients may produce confusing results during analysis of WGL specimens. Likewise, subjects may, for various reasons, fail to continue drinking the lavage solution.

Although WGL technique is a non-invasive, safe and useful method for obtaining intestinal secretions for use in clinical research into gut immunity, it is still, at this stage, not a practical method to be used for clinical diagnosis. Furthermore, WGL is not suitable for use in large-scale field studies. WGL is contraindicated in cases suspected to have intestinal obstruction. The salty taste of the lavage solution used in my research rendered the solution not very palatable for some subjects and may cause nausea and/or abdominal discomfort. However, the above symptoms were experienced only by one (out of 25) healthy volunteer and few elderly patients. An experienced nurse is required to supervise the intake and output of the lavage fluid to encourage subjects, particularly the elderly, to continue drinking the lavage

solution at a regular rate and to make sure that only clear fluid stools passed per rectum will be collected.

Generally, WGL technique was found to be a useful method for obtaining intestinal fluid which is essentially a gut perfusate for research into gut immunity and may have potential for clinical applications.

Section 2

INTESTINAL IgA, IgM AND IgG IN INFLAMMATORY BOWEL DISEASE AS COMPARED TO HEALTHY VOLUNTEERS

Concentrations of immunoglobulins, particularly TIgA as the predominant immunoglobulin in the intestinal secretions, have been measured directly in intestinal fluids obtained by WGL technique or intestinal intubation, or even in faecal material. Because intestinal fluids are not easily accessible, investigators have used saliva as a material to study concentrations of secreted immunoglobulins (see chapter 2).

Previous research in the G.I laboratory (O'Mahony et al, 1990) investigated levels of immunoglobulins in WGL fluid obtained from patients with a variety of gastrointestinal diseases and compared these levels with levels of immunoglobulins in WGL fluid of normal controls who were described as "immunologically normal" patients (see below). In the above mentioned study, the mean value of the concentrations of TIgA in WGL fluid of the normal control was found to be 136.9 ±20.6 μg/ml with a range of 7.9-403. Compared with levels of TIgA in WGL fluid of patients with Crohn's disease (with a mean of 176.1±58.0 μg/ml), there were no significant differences between levels of TIgA in WGL fluid of patients with Crohn's disease and the normal control while levels of TIgM and TIgG in WGL fluid of patients with Crohn's disease were found to be significantly higher than those of the normal control. Similar results were obtained by Mwantembe (1992)

who found that the median of levels of TIgA in WGL fluid of normal controls (immunologically normal patients) was 143 μg/ml with a range of 12-478 with no significant differences between levels of TIgA in WGL fluid of normal controls and patients with IBD (either Crohn's disease or ulcerative colitis) while levels of TIgM and TIgG in WGL fluid of patients with IBD were found to be significantly higher than those of the normal control.

Studies on jejunal aspirates (Arranz, 1993) have shown that IgA is the predominant immunoglobulin followed by IgM and IgG and the median of the concentrations of TIgA in a group of normal controls was reported as 102.5 μg/ml with a range of 23-540.6. Based on the concept of the common mucosal immune system, the concentration of TIgA was measured in saliva (Barton et al, 1990) on the ground that salivary immunoglobulins may represent the humoral immunity in the gut. The median of levels of TIgA in pure parotid saliva was found to be 165.8 μg/ml with a range of 66.5-606.9. However, further studies showed that levels of salivary immunoglobulins were not consistent with levels of immunoglobulins in the gut (O'Mahony et al, 1991a).

Concentrations of TIgA have also been also measured in faecal material using single radial immunodiffusion (Haneberg and Aarskog, 1975) and ELISA (Ferguson et al, 1994). However, results obtained from analysis of faecal material in the former study were found to be unreliable because specimens were not treated with protease inhibitors and the assay used is relatively insensitive. Results

obtained from the latter study showed that levels of TIgA in faecal material contained only about 4% of the amount produced in the gut as compared with levels of TIgA in WGL fluid. Furthermore, O'Mahony and colleagues (1990) showed that faecally contaminated specimens obtained during the early stage of WGL contained much less TIgA (2.0-3.8 μg/ml) than did clear specimens (16.3-91.4 μg/ml). The low profile of TIgA in faecal material may be due to mechanical and/or biological interference with the immunoassays by substances present in the faecal material. Furthermore, the immunological findings in the faecal material may represent only the findings in the colon.

The above studies reported higher levels of TIgA than those found in WGL fluid of 22 healthy British volunteers cited in chapter 6 of this thesis (where the median was 73.25 μg/ml). Likewise, the ranges of levels of TIgA reported in the above studies were found to be more widely scattered than the range of the concentrations of TIgA in WGL fluid of the healthy volunteers (a range of 10.00-173.20). Furthermore, the previous studies on the concentrations of immunoglobulins in WGL fluid found no significant differences between levels of TIgA in IBD patients and the normal controls (O'Mahony et al, 1990; Mwantembe, 1992) while my research into the total immunoglobulins in WGL fluid of healthy volunteers and IBD patients showed that healthy volunteers had significantly lower levels of TIgA. The reasons for the differences between the findings of the previous studies and the findings of my research are described below. Subjects used as normal controls in the work of O'Mahony et al (1990) and Mwantembe (1992) were a mixture of

patients who had a diagnosis of irritable bowel syndrome (IBS) or constipation. Others had presented with a variety of gastrointestinal disorders but various clinical investigations failed to detect any abnormality. The aetiology of IBS is still unknown and patients with IBS are usually prescribed spasmolytic drugs the effect of which on gut immunity is unknown. Likewise, constipated patients are usually on laxatives the effects of which on gut immunity have not been revealed. Therefore, levels of immunoglobulins obtained from the above subjects may not be suitable to be used as normal controls. Subjects used as controls in my research were young (age ranged between 22 and 44 years) healthy volunteers.

The method of collecting and processing of WGL fluids as well as the ELISA used for the measurement of immunoglobulins in the lavage fluids have been markedly improved since the introduction of WGL technique into the G.I unit in 1988. As described above, WGL is now supervised by experienced nurses to encourage subjects to drink 200 ml of the lavage solution every 10-15 minutes and to ensure accurate recording of the volumes of the fluid intake and output as well as to make sure that only clear fluid passed per rectum is collected. The method of processing WGL fluid has also been technically improved and the volumes of protease enzymes inhibitors added to the lavage fluid have been adjusted to minimise the activity of these enzymes. Filtering WGL fluid through special filter paper rather than centrifuging it has resulted in shortening the time required for WGL fluid processing.

It seems likely that the above technical development in the methods of collecting, processing and assaying WGL fluid has produced more accurate results than those obtained in previous studies. Comparing the results of the analysis of intestinal secretions obtained by the current WGL technique and those obtained by invasive methods, such as intestinal intubation for obtaining jejunal aspirate or for segmental gut perfusion, the results of WGL seems to be comparable to the other methods. Furthermore, WGL has the advantage of being non-invasive and well-tolerated.

Although the median level of TIgA in WGL fluid obtained from healthy volunteers in my research were shown to be lower than the medians and means reported previously, yet the range of the concentrations of TIgA was found to be relatively widely distributed (between 10.00 and 173.20 μg/ml). Whether the wide range of the concentrations of TIgA is due to a real biological phenomenon or due to other unknown factors is not, as yet, known. Effects of age, nutritional status and smoking habit on gut immunity are possible factors that may have confounding effects on levels of intestinal immunoglobulins. The effect of smoking/non-smoking on gut immunity of healthy volunteers was investigated (see chapter 8) and it was found that smoking does not seem to be one of the factors responsible for the wide range of TIgA in healthy subjects.

The main aim of administering oral vaccines is to stimulate intestinal immunity, particularly IgA, against enteric pathogens. Therefore, an important implication of

the wide range of levels of TIgA in the gut is the effect of oral vaccination on intestinal immunity. This is because a given dosage of the oral vaccine that may stimulate intestinal immunity against enteric pathogens in some individuals may not do so in others. Studies on the effect of oral vaccines on gut immunity (chapter 6 and personal communication, Dr Åhren, University of Göteborg, Sweden) showed that individuals who had very low or high levels of IgA antibodies in intestinal fluids did not respond to the oral vaccine as did others with optimal baseline levels of IgA antibodies. Therefore, further research into the factors affecting levels of IgA in the gut considering the age and nutritional status of the individuals involved is necessary.

Section 3

THE INTESTINAL IMMUNE RESPONSES TO THE ORAL TYPHOID VACCINE Ty21a

Since the gut is the portal of entry of the enteric pathogens, it is hoped that oral vaccines will protect against these pathogens by stimulating the production of IgA antibodies, the major component of the mucosal immune system. Introduction of the oral poliovirus vaccine (OPV) has resulted in elimination of the paralytic disease from many advanced countries and this encouraged workers to examine the possibility of developing oral vaccines against other enteric pathogens. However, oral vaccination against *Salmonella typhi*, the causative organism of typhoid fever, has produced equivocal results. The protective efficacy of the oral typhoid vaccine Ty21a, given at dosages containing 10^9 live *Salmonella typhi* organisms, varied from 42 to 96% in Indonesia and Egypt respectively. Experimental studies have shown that the vaccine Ty21a, given at the same dosages to healthy subjects from a non-typhoid area, produced either unmeasurable (Forrest et al, 1991) or meagre intestinal immune responses (Bartholomeusz et al, 1986).

Field trials investigated the protective efficacy of the oral typhoid vaccine Ty21a in areas endemic with typhoid fever using different formulas, schedules and dosages of the vaccine. However, the baseline intestinal antibody status of subjects from typhoid-endemic areas may be different from that of subjects living in non-endemic areas and the protective efficacy calculated from these field trials cannot be

extrapolated to populations from non-typhoid areas. Therefore, it was important to investigate the effect of the oral typhoid vaccine Ty21a on the intestinal immunity of healthy volunteers from non-typhoid areas. Previous studies investigated the immune responses to the oral typhoid vaccine in serum and saliva (see chapter 2). However, there is now increasing evidence that the findings in serum and saliva do not necessarily reflect the findings in the gut and it will be misleading to extrapolate the findings in the systemic immune system to the gut.

Forrest et al (1991) investigated the intestinal immune responses to the oral typhoid vaccine Ty21a in healthy volunteers from a non-endemic typhoid area excluding subjects who had had natural typhoid infection or had been vaccinated against typhoid fever. The above study showed that peak intestinal antibody responses were produced 2-3 weeks after oral vaccination with Ty21a vaccine. The above findings were useful in determining the time at which the post-vaccination specimens of WGL fluid should be collected from the British healthy volunteers in my study.

WGL fluids, essentially gut perfusate, were obtained from 22 British healthy volunteers before and 3 weeks after oral vaccination with Ty21a vaccine. In my studies, WGL technique was found to be safe, non-invasive and well-tolerated. However, WGL technique may not be suitable for evaluation of intestinal immune responses in large populations for field trials. The oral typhoid vaccine Ty21a,

given at a dose of 2 X 10^9 viable *Salmonella typhi* Ty21a organisms in the form of enteric-coated capsules on alternate days was found to be safe and well-tolerated.

No significant differences were found between levels of TIgA, TIgM and anti-Rc mutant of *Salmonella typhimurium* IgA antibodies in WGL fluid of the healthy volunteers before and 3 weeks after oral vaccination with Ty21a vaccine. These findings indicated that changes in anti-*Salmonella typhi* LPS antibodies were antigen-specific and not due to a polyclonal immune response. Furthermore, the above findings support the hypothesis of WGL fluid as a perfusate (see chapter 4).

The significant increase in anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of 14 out of 22 (63.6%) healthy volunteers after vaccination with the oral typhoid vaccine Ty21a may be regarded as a relatively modest increase compared to the increase in the intestinal IgA antibodies (anti-LPS antibodies) in gut lavage fluid of 12 out of 13 (92%) healthy volunteers after administration of the oral cholera vaccine (a combination of B-subunit of cholera toxin and whole cell organisms) (Svennerholm et al, 1984b). The finding, in my study, of a modest increase in the intestinal immune responses against the oral typhoid vaccine Ty21a is consistent with a previous study (Bartholomeusz et al, 1986) which investigated the intestinal immune responses to the vaccine Ty21a, taken as 3 gelatine-coated capsules containing 10^9 viable organisms each, in jejunal aspirate. Serological studies investigating the immune responses against the oral typhoid vaccine Ty21a have also shown modest responses in serum. The seroconversion (IgG and IgM

antibodies, measured by ELISA) produced by the vaccine Ty21a, taken as enteric-coated capsules containing 10^9 live organisms, in adult healthy American volunteers was approximately 44% (Black et al, 1983).

Several studies investigated the possibility of using the oral typhoid vaccine Ty21a as a vector to carry other vaccines for the protection against other enteric infections such as *Shigella* and cholera. However, in the light of the above findings the use of Ty21a vaccine as a carrier for other vaccines may not be encouraged. Indeed, the use of the vaccine Ty21a as a carrier for a vaccine against cholera (*V. cholera* O1 serotype Inaba O antigen) produced only 25% protection and the use of Ty21a vaccine as a carrier for a vaccine against *Shigella* (*Shigella sonnei* O antigen) produced a wide range of protective efficacy (Morris et al, 1992). The modest immunogenicity of the vaccine Ty21a may be because it is too attenuated to survive long enough in the gut, to interact enough with lymphoid tissues for efficient stimulation of intestinal antibodies. More immunogenic but less attenuated vaccines may be more invasive and more likely to produce adverse reactions. Therefore, it seems that a balance is required between the optimal immune responses and the adverse reactions produced by a successful candidate vaccine. Another possible explanation for the modest immunogenicity of the vaccine Ty21a is the low dose of viable organisms per enteric-coated capsule. Forrest et al (1991) showed that there was a significant increase in the intestinal (as well as systemic) antibodies of healthy volunteers after oral vaccination with Ty21a vaccine when given at doses containing 10^{11} viable *Salmonella typhi* organisms per enteric-coated capsule but no

responses were obtained when the vaccine was given at doses containing 10^9 viable organisms.

Genetic factors that render some individuals restricted in their immune responses to the vaccine may also be considered during interpretation of the immune responses against Ty21a vaccine. In mice, studies showed that the mouse strain CH3/HeJ does not respond to some kinds of LPS (Forui and Coutinho, 1978). This was attributed to a genetic disorder causing the absence of specific receptors on macrophages and lymphocytes of the mouse strain CH3/HeJ resulting in unrecognition of some types of LPS.

The concentrations of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of 4 volunteers showed no significant changes 3 weeks after oral vaccination with Ty21a vaccine while the concentrations of these antibodies in WGL fluid of another 4 volunteers showed a significant decrease 3 weeks after vaccination. Subjects who had no changes or decrease in their intestinal antibodies after oral vaccination were mostly subjects who had very high or very low baseline intestinal antibody levels. These results are in accord with findings obtained by a Swedish group (personal communication, Dr. Åhren, University of Göteborg, Sweden) during research into the intestinal immune responses against oral cholera and *E. coli* vaccines.

The absence of significant increase in the levels of intestinal IgA antibodies in WGL fluid of subjects who had high baseline levels intestinal antibodies may indicate that the magnitude of the intestinal immune responses to the orally administered vaccine Ty21a inversely dependent on the pre-existing specific antibodies which had most likely been acquired, in subjects from non-endemic areas, through subclinical exposure to non-typhoidal *Salmonella* spp. sharing O antigens similar to those of *Salmonella typhi*. Forrest (1992c) showed that the presence of high levels of cross-reacting intestinal antibodies in healthy subjects significantly reduced the magnitude of local intestinal immune responses to the oral typhoid vaccine Ty21a. The blocking effect of the cross-reacting antibodies was attributed to the presence of high levels of anti-*Salmonella typhi* LPS IgA antibodies which prevented boosting of primary intestinal immune response by a further dose of Ty21a vaccine. Therefore, it seems that obtaining a satisfactory local immune response may requires a fine balance between immunological priming and the presence of cross-reacting antibodies.

Subjects who had very low levels of baseline intestinal antibodies failed to have a significant increase in the concentrations of the intestinal IgA antibodies against the oral typhoid vaccine Ty21a. These findings are consistent with previous work by Murphy et al (1991) who showed that, unlike school-age children and adults, young children (infants and toddlers) from a typhoid-endemic area (Chile) failed to respond to the oral typhoid vaccine Ty21a. These findings suggest that levels of pre-vaccination cross-reacting intestinal antibodies should be taken into

consideration in the interpretation of the immunogenicity of the vaccine Ty21a. Therefore, it seems that the presence of an optimal levels of pre-existing intestinal antibodies may be an important factor in determining the outcome of the intestinal challenge to a defined oral vaccine. Subjects who had a decrease in the levels of anti-*S. typhi* LPS IgA antibodies may have had their peak concentration of intestinal antibodies at a time earlier than the date of the post-vaccination WGL sampling.

There was an overall increase in serum antibody titres of IgA, IgM and IgG. However, this increase was not correlated with the increase or decrease in the intestinal antibody titres in the individual subjects. The above findings are consistent with the increasing number of studies showing that systemic and intestinal immune systems are separate, but related, systems. It is now well-established that although serum antibodies are commonly detected after oral vaccination or enteric infection, including non-invasive infections, determination of the concentrations of serum antibody titres and local intestinal antibody titres are poorly correlated. In addition, Forrest et al (1992) showed that priming with parenteral vaccination, using killed typhoid vaccine, neither enhanced nor suppressed the subsequent intestinal IgA immune responses to a booster course of the live oral typhoid vaccine Ty21a. Therefore, it seems that levels of serum antibodies are dissociated from levels of intestinal antibodies and that serum antibodies may not play an important role in the protection against enteric infection.

The quantitative studies of the antigen-specific antibodies in WGL fluid (as well as in serum) using the modified ELISA developed in this research seems to be a satisfactory approach for the evaluation of the local intestinal immunity and to investigate the effect of orally administered vaccine on gut immunity. The specificity of the ELISA method used in this research was investigated by testing the WGL fluid of the volunteers before and 3 weeks after vaccination against Rc mutant of *Salmonella typhimurium*. As cited above, the absence of significant differences between levels of anti-Rc mutant of *Salmonella typhimurium* IgA antibodies in WGL fluid of the healthy volunteers before and 3 weeks after vaccination (while there was significant difference between levels of anti-*Salmonella typhi* LPS IgA antibodies before and 3 weeks after vaccination) indicated that changes in antibody titres after vaccination with Ty21a vaccine were specific and not due to a polyclonal up-regulation of the intestinal immune system.

In *Salmonella*, as in other Gram-negative bacteria, LPS is regarded as a major antigen in the outer membrane of the cell wall (Jawetz et al, 1987). The O-somatic polysaccharide side chains (O antigen) of LPS is known to be an important factor involved in the virulence of many Gram-negative bacteria, particularly of *Salmonella* (Hammond et al, 1984; Eisenstein, 1975; Svenson and Lindberg, 1979; Collins, 1974; Levine et al, 1983). Strains of *Salmonella typhimurium* lacking the complete O antigen (rough forms) were found to be less virulent than strains with complete LPS structure (smooth forms) (Hammond et al, 1984). The loss of O antigen from LPS of *Salmonella typhimurium* mutants resulted in an increase in the

ingestion of these mutants by macrophages when compared to parent strains (with complete LPS structure) both *in vivo*, as measured by the clearance of bacteria from the blood, and *in vitro*, as measured by the uptake of these strains by mouse intraperitoneal macrophages (Hammond et al, 1984). Uptake of bacteria by phagocytic cells requires activation of complement by the alternative pathway, a process involving interaction between C3b macromolecules on the bacterial surface and C3b receptors on the macrophages (Roitt et al, 1985; Roitt, 1994). Since strains of *Salmonella typhimurium* with complete LPS are relatively resistant to phagocytosis, it has been suggested that O antigen is involved in the resistance to ingestion by macrophages by covering the bacterial cell with a surface layer which does not allow the products of the complement on the bacterial surface to interact with C3b receptors on the macrophages, thus avoiding phagocytosis (Hammond et al, 1984; Roitt, 1994).

In animals, immunisation of rabbits with O antigen obtained from different *Salmonella* serotypes resulted in stimulation of anti-O antigen antibody titres nearly as high as those elicited by injection of heat-killed whole cell vaccine (Svenson et al, 1979; Svenson and Lindberg, 1981). In the above studies, anti-O antigen antibodies produced in rabbits protected mice against experimentally induced mouse typhoid (by intraperitoneal injection of *Salmonella typhimurium* SH 2201). Deletion of O antigen from LPS of gal E mutants of *Salmonella typhimurium*, used as an experimental vaccine, resulted in the loss of the protective efficacy of the vaccine against pathogenic strains of *Salmonella typhimurium* injected

intraperitoneally in mice (Germanier, 1970; Germanier and Fürer, 1971). The importance of LPS in the pathogenesis of some other Gram-negative bacterial infections is well-documented (Levine et al, 1983; Svennerholm, 1975; Svennerholm et al, 1975; Kopecko et al, 1980). LPS of *V. Cholerae* has been reported to be an important factor involved in the pathogenesis of cholera infection (Levine et al, 1983) and it has been shown that anti-*V. Cholerae* LPS antibodies provided animals with protection against experimentally induced cholera (Svennerholm, 1975; Svennerholm et al, 1975). In *Shigella*, the importance of LPS as one of the virulence factors is best illustrated by the phase variation shown by *Shigella sonnei*. *Shigella sonnei* phase I have the complete LPS structure and are usually virulent while *Shigella sonnei* phase II have incomplete LPS (without O antigen) and are non-pathogenic (Kopecko et al, 1980). In the above study, variation of *Shigella sonnei* between phases I and II was associated with the loss of a large plasmid known as 120-Mdal (120 Megadaltons in size).

The above studies highlighted the importance of LPS of *Salmonella* (and some other Gram-negative bacteria) as a major factor involved in the virulence of Gram-negative bacteria and have suggested that anti-LPS antibodies provided animals with serotype-specific protection against experimentally induced infection. However, mice are not the definitive host of *Salmonella typhi* and humans are the only known host of the organism. Furthermore, the exact pathogenesis of typhoid fever is not well-understood. Therefore, the findings in the above studies may only be applicable to animals.

Since the oral typhoid vaccine Ty21a lacks the capsular antigen (Vi antigen) (Germanier and Fürer, 1975; Gilman et al, 1977), measurement of antibodies against Vi antigen would not be relevant. Porin antigens of the outer membrane of the cell wall of *Salmonella* are not subspecies-specific (Svenson et al, 1979) and high titres of cross-reacting antibodies against porin antigens of *E. coli* may be obtained (personal communication, Dr. I Poxton, University of Edinburgh).

Because the precise mechanism by which the oral typhoid vaccine Ty21a may protect against typhoid fever is not, as yet, well-understood, the intestinal immune responses against other *Salmonella* antigens should be investigated. For instance, the flagella of *Salmonella typhimurium* were reported to be an important virulence factor in the murine typhoid model (Carsiotis et al, 1984). In an *in vitro* macrophage assay, flagellated salmonellae survived within macrophages more than did non-flagellated organisms (Weinstein et al, 1984). Since the oral typhoid vaccine Ty21a possesses the flagellar antigen (H antigen) (Gilman et al, 1977), investigation of the presence of anti-*Salmonella typhi* H antigen antibodies in the intestinal secretions, using WGL technique, may provide further information about the intestinal immune responses against the vaccine.

I would like to emphasise the fact that my research into the effect of the oral typhoid vaccine Ty21a on intestinal immunity involved the quantitative measurements of intestinal immunoglobulins and antigen-specific antibodies as parameters of the intestinal immune responses against the vaccine and not as

parameters of the protective efficacy of the vaccine. This is because the correlation between the magnitude of the intestinal immune responses and the clinical protection against infection has not, as yet, been established.

Since better understanding of intestinal immunity may help in developing vaccines against enteric infections, the findings in this study may have important implications for the oral vaccination programmes. Because the main target of vaccination programmes is the control of infectious diseases in countries of the developing world where subjects are under continuous exposure to a wide spectrum of intestinal pathogens, further research into the intestinal immune status of these subjects is required. Intestinal immunity together with the nutritional status may be important determinants of the outcome of vaccination programmes.

As cited above, the precise mechanism(s) by which the intestine is protected against the wide range of intestinal pathogens is(are) not, as yet, well-understood. In addition to humoral intestinal immunity, cell-mediated immunity has been suggested to play an important role in the protection against enteric pathogens, such as *Salmonella typhi* and *Shigella* infections. Intestinal infection with *Salmonella typhi*, *Shigella* or oral vaccination with Ty21a has been found to induce IgA-dependent cellular cytotoxic activity (Tagliabue et al, 1984). Cytokine-mediated cellular immunity has also been suggested to play a role in the protection against *Salmonella typhi* (Levine and Nataro, 1994). However, the above studies were carried out using peripheral blood and, as described before, the findings in

serum may not reflect the findings in the gut. Non-invasive methods for investigation of cell-mediated immunity in the gut are not available and the role of cell-mediated immunity in the protection against enteric pathogens remains to be elucidated.

Section 4

THE INTESTINAL IMMUNE RESPONSES TO NATURALLY-ACQUIRED SALMONELLA INFECTION

Bacterial infection of the gut lumen has been reported to prime the intestinal immune system to produce SIgA which is believed to provide specific immune protection for the gut to subsequent infection by a mechanism known as "immune exclusion". This mechanism involves the prevention of binding and colonisation of the mucosal surface by the causative organisms as well as the neutralisation of bacterial toxins. In addition, invasive organisms such as *Salmonella* and *Shigella* have been reported to stimulate IgA-dependent cellular cytotoxicity and the major histocompatibility (MHC) class I-restricted cellular cytotoxicity (Kauffmann, 1988). In my research into the effect of naturally-acquired salmonella infection on gut immunity, patients who had had salmonella infection were found to have measurable concentrations of intestinal anti-*S. typhi* LPS IgA (and IgM) antibodies in WGL fluid. However, there were no significant differences between levels of anti-*S. typhi* LPS IgA antibodies (expressed either as units/ml or units/mg of TIgA) in WGL fluid of patients who had had salmonella infection and those of healthy British volunteers.

The absence of significant differences between levels of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of patients who had had salmonella infection and those of the healthy volunteers may be due to the relatively long period between

the date of infection and that of WGL sampling. This was mainly due to practical difficulties in recruiting the above patients. Therefore, the peak of the intestinal immune responses, when there had been responses, may have passed so that the levels of the intestinal antibodies found in WGL fluid of the above patients did not represent the actual values of the intestinal antibodies induced by the infection.

The absence of significant differences between the concentrations of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of patients who had had salmonella infection and those of the healthy volunteers may also be due to low affinity of the immune responses of the above patients. As cited before, studies in mice showed that a genetic disorder may result in the absence of LPS-specific receptors on the macrophages and lymphocytes. The lack of interactions between antigen-presenting cells and LPS may result in low affinity immune responses. However, findings in animals cannot always be extrapolated to man and whether genetic factors are responsible for low profile immune responses in man or not is not, as yet, clear.

Another possible explanation for the absence of significant differences between levels of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of patients who had had salmonella infection and those of the healthy volunteers may be the presence of relatively high baseline levels of cross-reacting intestinal antibodies in the healthy volunteers. This may be due to the exposure of the healthy volunteers

to a previous infection (either overt or subclinical) with non-typhoidal *Salmonella* spp (salmonellosis).

The relatively small sample size of patients who had had salmonella infection (n=8) may be another explanation for the absence of statistically significant differences between levels of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid of these patients and those in WGL fluid of the healthy volunteers.

Typhoid fever has been reported to stimulate both intestinal and systemic, humoral and cell-mediated, immune responses. Specific intestinal SIgA antibodies are thought to play an important role in the prevention of mucosal penetration by *Salmonella typhi* organisms during the infection. Therefore, it was surprising to find out that one patient who had had typhoid fever had very low anti-*Salmonella typhi* LPS IgA antibodies expressed either as units/ml or units/mg of TIgA. Furthermore, the above patient was found to have the minimum value in the range of TIgA of patients who had had salmonella infection. The above findings may suggest that infection with typhoid fever does not always result in the induction of significant levels of specific intestinal IgA antibodies against *Salmonella typhi*. They may also have important implications for the vaccination programmes against typhoid fever because typhoid vaccines, particularly oral vaccines, are expected to stimulate intestinal IgA antibodies.

Although there have been many descriptive studies of the immune responses associated with *Salmonella typhi* infections or after immunisation against typhoid fever, yet these studies mostly described the immune responses in serum, IgA-secreting cell in the peripheral blood or in faeces. As discussed before in this thesis, the use of serum, peripheral blood lymphocytes (PBLs) or faeces to investigate intestinal immunity may not be valid.

Although intestinal IgA antibodies have been reported to appear within one week after enteric infection and as early as 3 days after re-immunisation (Svennerholm et al, 1984a), the duration of the presence of these antibodies in the intestinal secretions has not been investigated before. My study investigated the presence of intestinal antibodies against *Salmonella typhi* LPS for a period of up to 12 months after salmonella infection. However, because the optimal time at which the peak of the intestinal immune responses appear after naturally-acquired salmonella infection is not known and also, as cited above, because WGL sampling may have been obtained 'too late' to study the intestinal immune responses against *Salmonella typhi* LPS, the peak of the intestinal immune responses may had been "missed".

The antigen against which the WGL fluids were tested was LPS of *Salmonella typhi* which belongs to group D in the Kauffmann-White classification of the genus *Salmonella*. WGL fluid of patients who had had infection with *Salmonella enteritidis* (which also belongs to group D) may contain antibodies against two O antigens shared with *Salmonella typhi*. These antigens are O antigen factor 9 and O

antigen factor 12. Therefore, it is conceivable that antibodies measured in WGL fluid of patients who had had *Salmonella enteritidis* infection were more likely to be cross-reacting antibodies. The main reason for including patients who had had non-typhoidal with those who had had typhoidal salmonella infection was the small number of patients who could be recruited for the study. Given that the group contained a mixture of patients who had had either typhoidal or non-typhoidal salmonella infection, investigation of intestinal immune responses against a more common antigen such as Rc mutant of *Salmonella typhimurium* might have been more appropriate. In future work, a range of bacterial antigens (e.g. of bacteria not found in the gut) should be used. I would like to emphasise that the aim of this study was to measure intestinal immunoglobulins and antigen-specific antibodies in WGL fluid of patients who had had naturally-acquired salmonella infection as a parameter of intestinal immune responses and not as a direct evidence of the presence of protective immunity in the gut.

It was important to examine the intestinal immune status of healthy individuals from a non-endemic area because these individuals may have been infected with one or more serotypes of non-typhoidal *Salmonella* (salmonellosis) due to food poisoning and their intestinal antibody status may have never been investigated. Investigation of the intestinal antibody status in the above subjects is important because, as mentioned in the previous section, levels of pre-existing antibodies may be an important determinant of the outcome of vaccination against enteric infections.

Patients with IBD are known to have polyclonal up-regulation of intestinal immunoglobulin production. However, the presence of pre-existing antibodies against *Salmonella typhi* LPS has not been investigated before in such patients. Therefore, it was important to investigate the presence of antigen-specific intestinal antibodies in WGL fluid of IBD patients and to compare levels of these antibodies with those of healthy volunteers. The presence of significant differences between levels of TIgA in WGL fluid of IBD patients and those of patients who had had salmonella infections may be due to the fact that patients with IBD have, for unknown reasons, high levels of intestinal (and systemic) immunoglobulins.

There was no correlation between systemic and intestinal immune responses of patients who had had salmonella infection. These findings are consistent with the findings in the previous chapters of this thesis as well as with the findings of many other studies (see chapter 2).

Generally, the findings in this study indicated that there is a great need for further studies into the intestinal immune responses of patients who have had natural infection with enteric pathogens with a more careful approach into the optimal time at which intestinal antibody levels are likely to peak. Such studies should consider the wide range of levels of the pre-existing intestinal antibodies described in this study. In my study, WGL fluid was used as material to study gut immunity in British healthy volunteers, patients who had had salmonella infection and patients with IBD, and WGL technique was well-tolerated by all subjects involved in the

study. Therefore, the use of WGL technique, to obtain intestinal secretions, and the modified ELISA described in this thesis to study antigen-specific antibodies would make studies on gut immunity after enteric infections more feasible.

Section 5

EFFECT OF CIGARETTE SMOKING ON GUT IMMUNITY: DOES SMOKING SUPPRESS INTESTINAL IMMUNE RESPONSES ?

Tobacco smoking has been shown to modify the primary and secondary immune responses in animals and humans and has been suggested to influence the clinical course of inflammatory bowel disease (IBD) (see chapter 2). Nevertheless, most of the studies have investigated the effect of cigarette smoking on the systemic rather than the mucosal immune system. The effect of cigarette smoking has also been studied in pure parotid saliva and it was found that levels of salivary IgA of smokers is significantly lower than those of non-smokers or ex-smokers (Barton et al, 1990). However, further studies on saliva, in comparison with WGL fluid, showed that the immunological findings in saliva do not necessarily reflect those of the gut.

Therefore, investigating directly the effect of cigarette smoking on the intestinal mucosal immune system had become necessary due to a variety of reasons described below. Any modification of the mucosal immune system of the gut, by cigarette smoking, could have considerable implications on the intestinal immunity against enteric infections as well as on the efficacy of oral vaccines. Furthermore, cigarette smoking is an increasingly common habit in third world countries where malnutrition and poor health care standard are already major health problems.

The relationship, if any, between cigarette smoking and the clinical course of ulcerative colitis will also have important implications for the disease management. For instance, administration of nicotine to patients with ulcerative colitis may help in the management of this disease.

One study investigated the effect of cigarette smoking on the intestinal immune responses against three gut commensals (*Escherichia coli*, *Candida albicans* and *Proteus mirabilis*) using WGL in healthy subjects and patients with ulcerative colitis. However, the smokers in the above study had smoked a small number of cigarettes per day for a relatively short period of time (one year). Therefore, it was important to examine the effect of heavy smoking on gut immunity in young healthy subjects who had smoked >20 cigarette/day for more than three years comparing the results with those of young healthy non-smokers.

The results obtained in my study indicated that smoking did not suppress levels of intestinal IgA, IgM or antigen-specific IgA antibodies (anti-*Salmonella typhi* LPS IgA) as compared to the results obtained from non-smokers. Furthermore, when the oral typhoid vaccine Ty21a was used to induce gut immunity of both smokers and non-smokers, there was no significant difference between levels of anti-*Salmonella typhi* LPS IgA antibodies in WGL fluid (as well as in serum) of both groups. These findings exclude cigarette smoking as an important factor that may affect the magnitude of the intestinal immune responses to the oral vaccines and enteric infections. Therefore, other factors such as age and the nutritional status

may be important in determining the outcome of an antigenic challenge to the intestinal immune system. Such factors are to be investigated further.

The above findings may also indicate that the reported modification of the clinical course of ulcerative colitis in smokers is not mediated through suppression of intestinal immunoglobulins and antibodies. Possible mechanisms of how cigarette smoking might influence the clinical course of ulcerative colitis are described below.

Cigarette smoking has been reported to significantly decrease rectal blood flow in a group of patients with ulcerative colitis as compared to healthy non-smokers (Srivastava et al, 1990). In the above study, rectal blood flow was found to be inversely related to the concentration of nicotine in plasma. A decrease in rectal blood flow during smoking may be due to nicotine-induced vasoconstriction. A decrease in blood flow will result in a decrease in the amount of inflammatory mediators, such as prostaglandin E_2 (PGE_2) and leukotriene B_4, reaching the mucosal surface with the subsequent decrease in free radicals (extremely reactive chemicals) and tissue damage.

Another possible mechanism that may be involved in the protection of cigarette smokers against ulcerative colitis is the increase in colonic mucous production reported to be found in smokers. Non-smokers with ulcerative colitis were found to have reduced amounts of colonic mucous as compared with non-smokers controls

while patients with ulcerative colitis who did smoke had a similar rate of colonic mucous production to that of the controls (Cope et al, 1986). Increased mucous production in the colon will improve the mucous barrier function, an important protective barrier on the intestinal mucosal surface.

Finally, smoking has been found to decrease the intercellular junction of the intestinal epithelium enforcing the effectiveness and integrity of an important line of the mucosal defence beneath the mucous layer. Therefore, cigarette smoking may 'tighten' the intestinal epithelium.

The effect of cigarette smoking on the cellular aspect of the intestinal mucosal immune system has not been investigated. Systemically, cigarette smoking has been found to produce pronounced effects on cell-mediated immunity with a marked increase in the leukocytic count "smokers leukocytosis". The polymorphonuclear leukocyte count in cigarette smokers has been reported to be increased by up to 44% and the total number of T lymphocytes in the peripheral blood of smokers has also been found to be increased with a significant increase in $CD4^+/CD8^+$ ratio (Tollerud et al, 1989). The peripheral blood monocyte count in smokers has also been reported to be increased and the cells are defective in their capacity to kill intracellular *Candida* (Nielsen, 1985). Furthermore, heavy smokers have been found to have depressed natural killer cell activity and reduced antibody-dependent cellular cytotoxicity (Ferson et al, 1979).

In the respiratory tract, cigarette smoking has been found to increase significantly the number and secretory function of the alveolar macrophages (Harris et al, 1970) while in the cervical mucosa cigarette smoking was reported to produce a significant decrease in the number and activity of Langerhan's cells, which are important antigen-presenting cells involved in several chronic inflammatory conditions (Barton et al, 1988).

Therefore, further research into the effect of cigarette smoking on the antigen-presenting cells, such as macrophages, as well as on other cells in the gut may help in understanding the mechanism(s) by which cigarette smoking may affect the intestinal mucosal immune system.

Section 6

INTESTINAL MUCOSAL IgA DEFICIENCY

IgA deficiency is commonly defined as low levels or absence of this immunoglobulin class in serum. Subjects with IgA deficiency are also known to have very low numbers (or even absence) of IgA-producing cells in the intestinal mucosa, often associated with a "compensatory" increase in IgM-producing cells (see chapter 2). However, the paucity or absence of IgA in the intestinal secretions of subjects who have normal levels of IgA in serum has not been described before. Possible reasons that might have hampered previous recognition of intestinal IgA deficiency are described below.

Levels of immunoglobulins have always been measured in serum assuming that the findings in serum may reflect the findings in the gut. Measurements of the concentrations of intestinal immunoglobulins have been hampered by the difficulty in obtaining intestinal secretions. Previous studies reported wide ranges of intestinal IgA levels: the actual data showed the presence of very low levels of or even absent IgA in the intestinal secretions but these findings were ignored by the investigators presumably because they thought that these findings occurred due to technical errors or inadequate sampling.

The availability of non-invasive methods, such as WGL, for obtaining intestinal secretions as material in which to study gut immunity has increased the number of

subjects involved in research into gut immunity. Furthermore, the increasing use of WGL in cleansing of the bowel as a preparation for barium enema, colonoscopy or colon surgery as well as in the treatment of intractable constipation has increased the number of WGL specimens available for research into the intestinal mucosal immune system.

Since there was no standard range for the concentration of IgA in the intestinal secretions and because of the wide range of TIgA found in WGL fluid and in jejunal aspirates of subjects involved in my research into intestinal IgA deficiency, determination of the minimal levels of TIgA below which specimens were regarded as IgA deficient was based on the minimal value in the range of TIgA in WGL fluid of the 22 healthy volunteers (10-173 μg/ml) described in chapter 6.

Out of 391 WGL specimens in which concentrations of TIgA were measured, 6 specimens (1.5%) were found to have concentrations of TIgA ranged between zero and 5.2 μg/ml and were from subjects known to have normal levels of IgA in serum. One more subject was found to have no TIgA in WGL fluid but was also found to have hypogammaglobulinaemia.

Counting of IgA-producing plasma cells in the lamina propria of the above mentioned 6 cases revealed the presence of 2 different sets of subjects. There were subjects who had low levels of IgA in WGL fluid but counting of IgA-containing cells in the lamina propria of intestinal biopsies from these subjects revealed the

presence of numbers of plasma cells that fell within the range of plasma cells in the normal controls. The other set contained subjects who had low levels or absent IgA in WGL fluid and very low numbers of IgA-containing cells in the intestinal biopsies i.e. these subjects had a true intestinal IgA deficiency as shown in WGL fluid and IgA cell counting.

The discrepancy between the findings in WGL fluid and the intestinal biopsies in the first set of data may be due to technical error in collecting or processing of WGL fluid which resulted in false negative results. As described in chapter 4, analysis of PEG contents in sequential lavage samples of one patient involved in the study showed that some samples had very low contents of PEG and further investigation showed that these samples were contaminated (diluted) with urine.

Another possible technical error that might have occurred is collecting faecally contaminated rather than clear fluid WGL specimens. Analysis, by Dr. Croft (a research fellow), of faecally contaminated WGL specimens collected from constipated children revealed the presence of low IgA levels in these specimens. Deliberate addition, by some children, of pure lavage solution into the WGL specimens resulting in marked dilution and false low levels or negative IgA was also been found to be one of the difficulties encountered during WGL in children.

It is conceivable, although unlikely, that those cases with low levels or absent IgA in WGL fluid but with a normal IgA-producing cell count had a defect in the

transport of polymeric IgA into the external secretions due to deficiency in secretory component (SC) (see below). Therefore, if repeated measurements of IgA levels in WGL specimens of subjects with normal numbers of IgA-producing cells in the lamina propria revealed the presence of low levels of IgA in these specimens then the transport system of polymeric IgA into the intestinal secretions should be investigated further.

The subject with hypogammaglobulinaemia (who had absent IgA in WGL fluid) was found to have no IgA-producing plasma cells in duodenal biopsy. This result validated the technique used for counting plasma cells in the biopsies of the other subjects involved in this study.

One of the difficulties encountered in this study was finding relevant biopsies for the subjects who were found to have low levels or absent IgA in WGL fluid. Although most of the subjects had had duodenal biopsies taken at dates close to the dates of WGL fluid collection, the duodenal biopsy of one subject was found to be old (in relation to the date of WGL) and this biopsy may not have been relevant to the findings in the WGL fluid. Therefore, jejunal biopsies that corresponded closely in date to the dates of the WGL fluid were used for plasma cell counting. Nevertheless, immunohistochemical studies (Brandtzaeg, 1994) showed that duodenal and jejunal mucosa contain the same percentage of IgA, IgM and IgG-producing plasma cells (79, 18 and 3% for IgA, IgM and IgG respectively).

Another subject was found to have an old duodenal biopsy but no jejunal biopsy available for plasma cell counting. However, a colonic biopsy had been taken at a date close to the date of WGL fluid collection and it was used for the plasma cell counting. A third subject who had no IgA in WGL fluid was found to have had no relevant intestinal biopsy taken at a date close to the date of WGL fluid collection. The above subject was a 91-year old lady with active severe perianal Crohn's disease and intractable constipation. Therefore, obtaining an intestinal biopsy from the above lady was considered to be practically difficult. It is likely that the absence of IgA in the intestinal fluid of the above lady was due to the specimens being faecally contaminated (see above).

Due to ethical and practical reasons, one of the difficulties that faces researchers into gut immunity is obtaining relevant biopsies from healthy volunteers. Therefore, the biopsies used in my study as normal controls were duodenal biopsies from young subjects (aged <50 years) who had presented with a variety of gastrointestinal disorders but in whom various investigations had detected no abnormality. The number of IgA plasma cells in the biopsies of the above subjects varied between 255 and 948 cells/mm^2 lamina propria.

Out of 6 subjects with absent or near absent IgA in WGL fluid, 2 subjects were found to have 9 and 19 IgA-producing cells in duodenal biopsies. These 2 subjects were patients who presented with abdominal pain associated with loose stool or diarrhoea. Whether the intestinal and cellular findings of the above 2 subjects were

related to their unexplained abdominal pain and diarrhoea or not, is not clear. Considering the role of intestinal IgA in protection against a wide range of intestinal pathogens and considering the balance that should be maintained between intestinal IgA and the normal microbial flora to avoid compromising the mucosal barrier of the gut then one can assume that the immunological findings in the gut may be relevant to the clinical presentation of the above 2 subjects.

IgA2 is known to be more resistant to IgA proteases and has more capacity for agglutination of Enterobacteriaceae than IgA1. Furthermore, the immune exclusion mechanism has been reported to be suppressed in subjects with selective IgA deficiency, and gut opportunistic infections seen in AIDS patients have been suggested to be due to low levels of intestinal IgA (Brandtzaeg et al, 1991). However, the relationship between the quantitative measurements of the intestinal IgA and the clinical protection has not been well established.

One more subject who had no IgA in WGL fluid and was found to have no IgA-producing cells in a duodenal biopsy had a similar clinical presentation to the above 2 subjects but also had diabetes mellitus and hypogammaglobulinaemia.

There were discrepancies between the results obtained from measurements of IgA in jejunal aspirate and those of IgA plasma cells in the jejunal biopsies of subjects who had low levels or absent IgA in jejunal aspirate. Apart from 3 subjects (one had selective IgA deficiency), subjects who had low levels of IgA in jejunal

aspirates were found to have numbers of IgA plasma cells that fell within the range of (or were even higher than) the normal control. Furthermore, the number of IgA plasma cells in the jejunal biopsy of one control who had normal levels of IgA in jejunal aspirate was found to be very low (17 cells/mm^2 lamina propria). The above findings may indicate that the specimens of jejunal aspirate used in this study for the measurements of IgA were not reliable. As described before, jejunal aspirate used in this study might have been markedly diluted, due to a technical error (see page 261), during collection resulting in a false low levels or even absence of IgA measured in these specimens (jejunal aspirate collection was not one of the protocols of my study). However, the technique of collecting jejunal aspirate specimens has now been revised to exclude this technical error.

Three subjects who had no IgA in their jejunal aspirates were found to have no IgA-producing plasma cells in the lamina propria of their jejunal biopsies. These three subjects had presented with abdominal pain with diarrhoea. One subject had selective IgA deficiency and non-insulin dependent diabetes mellitus, but various investigations carried out on the other two revealed no abnormality. As cited above in cases who had similar clinical presentations and low levels of IgA in WGL fluid, the relationship, if any, between the immunological findings and the clinical presentation is not clear.

The presence of subjects with no IgA in their intestinal secretions but normal levels of IgA in serum has also been reported by researchers in Sweden (personal

communication, Dr. Åhren, University of Göteborg, Sweden). The above group described similar findings in a healthy staff volunteer during research into the intestinal immune responses against cholera and *E. coli* vaccines. Collaborative research into gut immunity, including the phenomenon of absence of IgA from intestinal secretions (in individuals who have normal levels of IgA in serum) between the Swedish group and researchers from the G.I laboratory of the University of Edinburgh is currently being carried out.

The mechanism by which intestinal IgA is secreted into the gut lumen involves a series of developmental stages of B cells resulting in differentiation, maturation and proliferation of these cells in the mucosa of the gut. Differentiation and maturation of B cells involves cellular and molecular pathways before plasma cells produce the polymeric IgA. Polymeric IgA then binds to the polymeric immunoglobulin receptor (PIgR) on the basolateral surfaces of the intestinal epithelial cells and is transported across these cells to be selectively secreted into the gut lumen attached to the SC as SIgA. The cellular pathway of B cell differentiation requires the presence of T cells, dendritic cells and stromal cells in Peyer's patches. Maturation of B cells requires the presence of transforming growth factor-β (TGF-β) and interleukin 4 (IL-4).

Antigenic activation of B cells occurs in the presence of interleukin 5 (IL-5) while interferon-γ (IFN-γ) may produce inhibitory signals resulting in down-regulation of IL-4. Final maturation of B cells and the clonal expansion of IgA-producing cells

require the presence of IL-2 and IL-6. J chain is required for the formation of polymeric IgA and SC is required for the active transport of IgA into the gut lumen. Therefore, a deficiency in one or more of the cytokines or a block in cellular pathways involved in the production of SIgA may result in its absence in the intestinal secretions.

Intestinal IgA deficiency is a newly discovered phenomenon that requires further investigation. Cases found to have IgA deficiency should be carefully examined with respect to nutritional status, previous infection and illness history, current and/or drug therapy and age. As described before in this thesis, smoking habit alone does not seem to be a factor that significantly affects gut immunity.

Section 7

Conclusion

There has been an increasing need for a method by which the intestinal immune system can be investigated directly, especially after the discovery that the intestinal immunity is independent from the systemic immune system such that the findings in the serum and other body fluids cannot be extrapolated to the gut. WGL technique has been shown to be a non-invasive, safe and reliable method for obtaining intestinal secretions in which to study gut immunity. The results of the serial lavage study have shown that WGL fluid is a perfusate rather than just a bolus of gut contents. Therefore, the above study validates the use of the first clear sample passed per rectum as a representative of the rest of the lavage effluent for use in clinical research into gut immunology and inflammation.

Although the WGL technique is useful in clinical research into gut immunology and inflammatory bowel disease, it is not a practical method to use for clinical diagnosis. The intake and output of the lavage fluid require close supervision by an experienced nurse throughout the procedure. This makes the WGL technique unsuitable for use in large-scale field studies.

Studies on the concentration of intestinal IgA in WGL fluid of patients with IBD and adult British healthy volunteers has shown, unlike previous studies reported from this centre, that levels of intestinal IgA in WGL fluid of patients with

ulcerative colitis or Crohn's disease, regardless of the disease activity, are significantly higher than those levels in WGL fluid of the healthy volunteers. While WGL fluid of the healthy volunteers contained small traces of TIgM and TIgG, levels of these immunoglobulins in WGL fluid of patients with IBD were significantly higher than those of the healthy volunteers. However, within the subgroups, levels of TIgA and TIgM in WGL fluid of patients with active disease (ulcerative colitis or Crohn's disease) did not differ significantly from levels of these immunoglobulins in WGL fluid of patients with inactive disease indicating that levels of IgA and/or IgM in WGL fluid cannot be used as indices of disease activity in patients with IBD. These results confirm the association of IBD with a major alteration in levels of intestinal immunoglobulins, particularly IgG. Whether the up-regulation of the intestinal immunoglobulins associated with IBD is due to leakage from the systemic immune system or due to increase in local production is not clear. Generally, reliable analysis of intestinal immunoglobulins of patients with IBD may provide important clues into the pathophysiology and immunopathology of IBD.

The oral typhoid vaccine, given at dosages of 2×10^9 live *Salmonella* organisms on 3 alternate days in the form of enteric-coated capsules, has been shown to be safe and well-tolerated by the vaccinees. However, the intestinal (and systemic) specific immune response produced by this vaccine, although statistically significant, is a modest increase if compared to the up-regulation of immune responses produced by other oral vaccines. These results are consistent with clinical experimental studies

investigating the intestinal immune responses against the vaccine in jejunal aspirate and in accord with recent large-scale field studies investigating the protective efficacy of the vaccine Ty21a. Therefore, the oral typhoid vaccine Ty21a may not be suitable for use as a vector to carry other vaccines against enteric infections. The absence of a significant correlation between the intestinal and systemic immune responses confirms that the intestinal immune system is quite separate from the systemic immune system.

Levels of intestinal polyclonal and anti-*Salmonella typhi* LPS IgA of patients who had had naturally-acquired salmonella infection did not differ significantly from levels of these immunoglobulins and antibodies in WGL fluid of British healthy volunteers. Furthermore, in one patient, infection with *Salmonella typhi* stimulated just meagre intestinal immune responses (both polyclonal and antigen-specific IgA). The relatively long period between the dates of infection and investigation may have resulted in missing the peak (if one existed) of the intestinal immune response against salmonella infection indicating that for future work the optimal timing of the study may be an important factor that may influence the outcome of the study. Low affinity of the immune responses of patients who had had salmonella infection, high baseline of cross-reacting intestinal antibodies, genetic resistance against LPS of *Salmonella* spp. and the small size of the sample involved in the study are other factors that may have resulted in the absence of a significant difference between levels of intestinal IgA in WGL fluid of patients who had had salmonella infection and the healthy volunteers. This study also showed that

patients with IBD have high levels of antigen-specific antibodies as compared to the healthy volunteers.

The findings in the studies on the oral typhoid vaccine and the intestinal immune responses against naturally-acquired salmonella infection may indicate that other factors such as the nutritional status, age and pre-existing intestinal cross-reacting antibodies should be considered during interpretation of the immune responses against oral vaccines and naturally-acquired salmonella infection.

Although studies on the effect of smoking on concentrations of IgA in saliva of smokers/non-smokers had shown low salivary IgA levels in smokers, studies on the effect of cigarette smoking on gut immunity of healthy volunteers indicated that smoking does not modify significantly the levels of intestinal immunoglobulins or specific antibodies against the oral typhoid vaccine Ty21a. Therefore, the protective effect, if any, of cigarette smoking reported to be found in patients with ulcerative colitis must be due to some other mechanism(s) than the levels of intestinal immunity. These mechanisms may involve an increase in colonic mucous production, decrease in rectal blood flow or a decrease in the para-cellular junctions of the intestinal epithelium. Further studies on the effect of cigarette smoking on the cellular aspects of the intestinal immune system are recommended.

Studies on WGL fluid and jejunal aspirate of patients and normal individuals collected during 1991/92 revealed the presence of a few subjects with very low

levels or absence of IgA in their intestinal secretions. These subjects were found to have normal levels of IgA in serum. Immunohistochemical studies on intestinal biopsies from the above subjects revealed the presence of only small numbers of IgA-plasma cells in the lamina propria of some of these subjects suggesting the presence of a previously unrecognised form of IgA deficiency. At this stage, the implications of intestinal IgA deficiency for gut immune function are not clear and the relationship between the clinical presentation of some subjects and this phenomenon is to be further investigated.

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Appendix

A copy of the information sheet used for the study of intestinal immune responses to the oral typhoid vaccine Ty21a

AN INFORMATION SHEET FOR THE STUDY OF INTESTINAL IMMUNE RESPONSES TO THE ORAL TYPHOID VACCINE Ty21a

Name: D.O.B: Sex:

Hospital: Unit No: Cosultant:

Date of study:

Session (pre/post-vaccination):

History of infection (if any):

Treatment given (if any):

History of travelling abroad:

History of vaccination (oral and/or parenteral):

History of other illness (Medical and/or Surgical):

Smoking habit (cigarettes/day):

Drinking habit (units/week):

Drug history (if any):

Family history:

Social history:

Results of laboratory investigations (pre/post-vaccination)::

(1) Total immunoglobulines

Serum: TIgG TIgM TIgA

Whole gut lavage: TIgA TIgM

(2) Anti-*Salmonella typhi* LPS antibodoies

Serum: IgG IgM IgA

Whole gut lavage: IgA IgM

(3) Anti-*Salmonella typhimurium* Rc-mutant IgA antibodies